TECHNICAL REPORT
NATICK/TR-17/017

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BACTERICIDAL COATINGS ON TEXTILES FOR REMEDIATION OF INTERMICROBE ACTIVITY (BaCTeRIA) SUMMARY REPORT

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July 2017

Final Report
October 2011 – September 2015

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
07/07/2017	Final	October 2011 – September 2015

4. TITLE AND SUBTITLE

BACTERICIDAL COATINGS ON TEXTILES FOR REMEDIATION OF INTERMICROBE ACTIVITY (BaCTeRIA) SUMMARY REPORT

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Tobyn A. Branck, Courtney M. Cowell, Jennifer M. Rego, and Robert Stote

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

U.S. Army Natick Soldier Research, Development and Engineering Center
ATTN: RDNS- TMS
10 General Greene Avenue, Natick, MA 01760-5020

8. PERFORMING ORGANIZATION REPORT NUMBER

NATICK/TR-17/017

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Development of antimicrobials that demonstrate targeted, specific activity against pathogenic strains of interest to the Army provides a promising strategy to protect the Warfighter from pathogenic threats where broad-spectrum antimicrobials may not be applicable. This technical report describes the culmination of work dedicated to the discovery, characterization, analysis and development of a narrow-spectrum antimicrobial that exhibits targeted activity against the surrogate strain of *Bacillus anthracis*, the microbial agent of anthrax. The antimicrobial agent investigated in this effort was a bacteriocin, a member of a class of antimicrobial peptides secreted by microorganisms to protect against competing microbes in the environment and was discovered from a screen of environmental isolates from Fort Devens. A cost-effective purification protocol was developed to yield sufficient qualities and purities of the bacteriocin to enable its future development. Preliminary investigations were conducted to evaluate the feasibility of synthesizing a multifunctional material with the antimicrobial peptide. Additionally, the production conditions of the bacteriocin from its producer organism was investigated to generate the optimal growth media for high quantities of bacteriocin production. The work cited in this report provides the foundation for future studies to further characterize and develop a novel application employing the bacteriocin in a construct that facilitates antimicrobial protection for the Warfighter.

15. SUBJECT TERMS

FIBERS	PATHOGENS	PATHOGENIC MATERIALS	ANTIMICROBIAL AGENTS
TEXTILES	PROTECTION	COST EFFECTIVENESS	ANTIMICROBIAL PEPTIDES
ANTHRAX	PURIFICATION	BACILLUS ANTHRACIS	ENVIRONMENTAL ISOLATES
BACTERIA	BACTERIOCINS	TARGETED ACTIVITY	MULTIFUNCTIONAL MATERIALS
COATINGS	MICROBIAL AGENT	PATHOGENIC THREATS	PATHOGENIC MICROORGANISMS
TARGETING	MICROORGANISMS	STRAINS(BIOLOGY)	NARROW-SPECTRUM ANTIMICROBIALS

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			Robert Stote
U	U	U	SAR	64	**19b. TELEPHONE NUMBER** *(include area code)* 508-233-4629

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Preface

This report documents work by the Biological Sciences and Technology Team (BSTT) of the Warfighter Directorate at the Natick Soldier Research, Development and Engineering Center (NSRDEC) during the period of October 2011 to September 2015. This work focused on the isolation, characterization and development of a narrow-spectrum antimicrobial agent to replace broad-spectrum antimicrobials for use in an array of applications. In Task I, the narrow-spectrum antimicrobial agents of interest, termed bacteriocins, were isolated from an environmental pool of bacterial isolates and characterized. The bacteriocin isolates were evaluated for bacteriocin-induced activity against three target microorganisms of interest to the military: *Bacillus anthracis* Sterne, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Of the over 100 environmental isolates collected, 11 were identified as possessing activity against *Bacillus anthracis* Sterne and *Staphylococcus aureus*, while no isolates were identified as being active against *Pseudomonas aeruginosa*. In Task II, a universal purification procedure was developed to rapidly harvest sufficient yields and purity of bacteriocins to facilitate their screening for activity. In Task III, a bacteriocin sample, termed bacteriocin 105b, that demonstrated consistent activity became and was readily produced. As a result, it became the focus of future studies. Building upon the work in the previous task, a purification protocol was established specifically for the purification of bacteriocin 105b. In Task IV, preliminary studies into the application of bacteriocin 105b in a multifunctional material were evaluated. The final task, Task V, was further focused on developing the optimal conditions for producing high yields of bacteriocin 105b to facilitate its use in applications of interest to the Army.

BACTERICIDAL COATINGS ON TEXTILES FOR REMEDIATION OF INTERMICROBE ACTIVITY (BaCTeRIA) SUMMARY REPORT

Introduction

The Biological Sciences and Technology Team (BSTT), Warfighter Directorate, of the Natick Soldier Research, Development and Engineering Center (NSRDEC) is investigating the use of narrow-spectrum antimicrobials for incorporation into textiles, using non-traditional methods such as biomimetic entrapment and electrospinning, to protect the Warfighter from pathogenic bacteria. This technical report summarizes work of the Bactericidial Coatings on Textiles for Remediation of Intermicrobe Activity (BaCTeRIA) effort from October 2011 to September 2015 within the BSTT to address the development of narrow-spectrum antimicrobial agents.

The need for the development of an alternative antimicrobial agent that could exhibit a narrow-spectrum of activity by only targeting specific strains of pathogenic bacteria has recently come into focus as strains of drug resistant bacteria are on the increase and the number of drugs being developed against them are decreasing (Cooper *et al*. 2011). This creates a situation where infections that are currently treatable may not be treatable in the very near future. Additionally, many of these drug resistant strains demonstrate resistance to the antimicrobials used in textiles, polymer surfaces, wipes and ointments, making the antimicrobials ineffective (Tattawasart *et al*. 1999, Thomas *et al*. 2000, McDonnell *et al*. 1999, Silver *et al*. 2003). Though a number of hypotheses have been offered to explain this increase in resistance, one of the leading causes has been identified as the use of broad-spectrum antimicrobials that unintentionally promote the selection of bacterial strains resistant to the antimicrobial (Levy *et al*. 2004). When broad-spectrum antimicrobials are employed, not all of the bacteria are killed. Those that have developed a resistance to the antimicrobial will survive. Since the more benign microorganisms that contribute to keeping drug-resistant pathogenic strains under control are killed off, the resistant strains are allowed to grow unfettered (Levy *et al*. 2004, Gulberg *et al*. 2011). After a few exposures to a broad-spectrum antimicrobial, the collection of bacteria becomes primarily composed of resistant strains, making the broad-spectrum antimicrobial ineffective. When this occurs, the current strategy is to replace an ineffective antimicrobial agent with another broad-spectrum antimicrobial agent. This provides an opportunity for the bacterial pool to be further screened for those bacteria that are now resistant to the new antimicrobial agent as well as the previous antimicrobial, leading to the development of multi-drug resistant bacteria, or “super bugs”. As concern about the emergence of multi-drug resistant bacteria increases, new strategies are being investigated to inhibit the development of resistant strains. One promising new strategy is to use narrow-spectrum antimicrobials, such as bacteriocins, as alternatives to broad-spectrum agents.

Bacteriocins are narrow-spectrum bacterial toxins secreted by bacteria to kill other closely related bacteria that are competing for the same resources. Many bacteriocins have very specific activity spectrums, as they only target one or two species. This selectivity offers a paradigm shift of applying antimicrobials where instead of broadly killing everything, specific pathogens could be targeted, leaving beneficial bacteria unaffected and capable of thriving (Abt *et al*. 2014). Generally considered a green alternative to the currently used antimicrobials, few

bacteriocins have demonstrated adverse effects on eukaryote cells (Cotter et. al., Cox et. al., Galvz et al.). These and other characteristics make bacteriocins an appealing option to supersede broad-spectrum antimicrobials.

The BaCTeRIA effort concentrated on isolating bacteriocins that exhibit activity against pathogenic microorganisms of interest to the Army, establishing the procedures to readily produce the antimicrobial agent(s), developing the techniques to entrap the bacteriocin on a textile and confirming its activity. This work was divided into several tasks, which are described in detail below.

Task I – Isolation, Characterization and Identification of Environmental Bacterial Isolates with Screening for Antagonism Against Three Bacterial Targets

To best identify bacteriocins that target pathogenic microorganisms of interest to the Army, a large pool of bacterial isolates collected from the environment was characterized. By screening such a large pool of isolates, it was possible to elucidate antimicrobial activity targeted at multiple relevant pathogens as well as investigate the specificity of the activity. Their activity was evaluated against three Army relevant pathogens: *Bacillus anthracis* Sterne, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Additionally, the isolated bacterial samples were characterized to establish their genus.

Task II – Protocol for Initial Purification of Bacteriocin

Bacteriocins show great promise to replace broad-spectrum antimicrobial agents in numerous applications due to the targeted activity of the bacteriocins. Their development in industrial applications has lagged due to the time consuming procedures implemented for the production and purification of bacteriocins. To successfully develop a bacteriocin for application as an antimicrobial agent in various platforms, a universal purification procedure is necessary. In this task, building upon an assessment of the current literature, a generic purification protocol was developed.

Task III – Development of Purification Protocol Specific for Bacteriocin 105b

From the initial evaluations of bacteriocins identified during Task I, one bacteriocin sample, termed bacteriocin 105b, was chosen for future aspects of the effort. Bacteriocin 105b was selected due to its strong activity demonstrated against *Bacillus anthracis* Sterne. A purification protocol was established to produce sufficient quantities of bacteriocin 105b with a desired purity to facilitate the use of this antimicrobial agent in applications that promote the development of non-traditional textiles that exhibit antimicrobial properties.

Task IV – Entrapment of Bacteriocin 105b onto Fabric with Titania

This task focused on initial evaluations of fabricating a multifunctional textile by incorporating antimicrobial and photocatalytic functionalities into a single textile through the encapsulation of bacteriocin 105b, by precipitation with titania, a photocatalytic matrix on a swatch of fabric. The resulting material is anticipated to exhibit both antimicrobial and photocatalytic properties. This work leveraged efforts of the Functional Oxides as Reactive Coatings for Enhanced Protection on Textiles (FORCE ProTex) effort, which investigated the entrapment of the bacteriocin nisin in titania. In this work, titania was precipitated in the presence of either a pure or semi-pure preparation of bacteriocin 105b on a swatch of fabric. The resulting swatch of bacteriocin

encapsulated titania composite was then evaluated for antimicrobial activity. Although entrapment of the semi-pure bacteriocin sample did result in a swatch that exhibited antimicrobial activity, the encapsulation pure bacteriocin did not, suggesting that the procedure for preparing the bacteriocin significantly impacts the resulting stability and activity of the bacteriocin. Further work must be conducted to assess and mitigate the inactivity of the pure bacteriocin for future use as well as evaluate the photocatalytic activity of the bacteriocin infused titania matrix on the development of a multi-functional material.

Task V – Optimization of Bacteriocin 105b Production in Defined Media

Because bacteriocin 105b specifically targets a pathogenic strain of bacteria of interest to the Army, this antimicrobial peptide holds great promise for application in antimicrobial platforms to protect the Warfighter. In order to realize the utilization of bacteriocin 105b in future applications, its production and purification processing must be optimized to generate a high yield of the peptide. In this task, several growth conditions for the bacteriocin 105b producer, *Bacillus subtilits* 105b isolated from Task I, were evaluated to determine the superior process for the production of bacteriocin 105b. Complex growth media as well as carbon and nitrogen sources present in the growth media were investigated. Upon conducting a crude cost analysis of growth media options, molasses media was pursued as offering a cheap, viable media for the production of bacteriocin 105b.

Task I - Isolation, Characterization and Identification of Environmental Bacterial Isolates with Screening for Antagonism against Three Bacterial Targets

Development of innovative, potent antimicrobials that exhibit activity against microorganisms of interest to the Army is necessary to protect from the increasing threat of biological warfare agents. Bacteriocins, a class of antimicrobial peptides that exhibits a narrow-spectrum of activity, provides an alternative to conventional broad-spectrum antibiotics to target Army-specific pathogenic threats. One approach to identifying potential novel bacteriocins of interest is to investigate bacterial isolates in the environment that produce bacteriocins to naturally defend against pathogenic bacteria. Readily available environmental isolates provide a large sample of potential valuable bacteriocins to be screened for activity against a desired target of interest as well as ascertain the spectrum of activity observed. Using this information, environmental isolates that display bacteriocin-driven activity against pathogenic bacteria can be identified for further characterization. The identification of a bacteriocin that exhibits specific activity against a pathogenic bacteria strain of interest is an important first step in the application of a unique antimicrobial agent to protect the Warfighter. This initial identification will lead to future work to optimize the production of the bacteriocin, the purification of the bacteriocin, and characterization of its activity and stability over time.

The purpose of this task was to generate a pool of microorganisms isolated from the environment and subsequently characterize and screen the pool for bacteriocin-driven antimicrobial activity against military relevant pathogenic target strains of bacteria. Environmental isolates were collected from several locations at Fort Devens. Identification of the environmental isolates was achieved via phenotypical analysis as well as analysis by the Omnilog system. Activity of the isolates was evaluated against three military relevant microorganisms: *Bacillus anthracis* Sterne, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The environmental isolates were screened against these targets for bacteriocin-like activity against the targets and the specificity of the activity was probed.

Isolation of Environmental Bacterial Isolates

Twelve samples of environmental bacteria were collected from surfaces at Fort Devens (Ayer, MA). Eight sites of interest were wiped with sterile cotton swabs, including AC ducts, a door handle, a cot, a cutting board, a kitchen sink and the handle of a washing machine. Nutrient agar plates were inoculated with the swiped cotton swabs. The plates were incubated at 37 °C for 24 h. Individual colonies were picked from each plate using a sterile loop and streaked onto a new nutrient agar plate to obtain a pure isolate. The initial plates inoculated by the cotton swab were then incubated again for another 24 h to permit any slow-growing colonies to develop. Newly developed colonies were picked and streaked onto a new nutrient agar plate to obtain a pure isolate.

Table 1 lists the surfaces swabbed, the assigned sample name and the total number of unique bacteria colonies isolated. Three unique surfaces investigated did not yield bacteria colonies: the tent canvas (FD1 and FD4), a door handle (FD5) and a scarcely used AC duct (FD9). The lack of bacteria colonies isolated from this door handle and AC duct may be due to the infrequent use

of both surfaces, which limited the interactions necessary for bacteria transfer from the environment to the surface. The absence of bacteria colonies isolated from the tent fabric may be due in part to the fact that the shelter was assembled only a month before the samples were collected. This short period of time may not have allowed sufficient exposure of the surface to the environment and people for microorganisms to be detected. Additionally, tent materials are treated with a water repellant and an antifungal treatment, which may inhibit the adhesion and/or growth of bacteria. Because the kitchen was recently set up and had not yet been used, it is expected that not many bacterial isolates would be present, which can be seen in the results. It is anticipated that re-visiting the kitchen to swab surfaces after the kitchen was used for meal preparation would yield different results, including the isolation of more microorganisms.

Table 1. Summary of Surfaces Probed for Environmental Bacterial Isolates

Sample Name	Surface	Number of Colonies Isolated
FD1	tent canvas	0
FD2	tent 6 cot metal handle	1
FD3	tent 6 AC duct return	2
FD4	tent canvas	0
FD5	door handle	0
FD6	tent 8 AC duct return	39
FD7	rigid shelter door handle	31
FD8	rigid shelter air return	24
FD9	AC duct return (barely used)	0
FD10	laundry 47 washer handle	9
FD11	kitchen sink and handles	7
FD12	kitchen cutting board	1

Identification of Environmental Bacterial Isolates

The environmental bacterial isolates were characterized. Identification of environmental isolates followed the flowchart from "Bergey's Manual of Determinative Bacteriology", which utilizes physical and biochemical analysis to identify the genus and species of unknown bacteria samples (Holt *et al*. 1994). These physical and biochemical tests included: (1) the Gram stain test, which is used to determine if the organism is Gram-negative or Gram-positive; (2) the acid-fast test, which identifies *Mycobacterium*; (3) the oxidase test, which identifies organisms that produce the enzyme cytochrome oxidase; (4) the catalase test, which differentiates *Staphylococci* (a catalase positive organism) from *Streptococci* (a catalase negative organism); (5) the starch hydrolysis test, which evaluates the organism's ability to synthesize and secrete enzymes to break down starch into smaller subunits to be used by the organism; (6) the mannitol and glucose fermentation test, which investigates how an organism metabolizes sugars; (7) the Voges-Proskauer test, which tests the organism's ability to produce non-acidic end products from the metabolism of glucose and is helpful in differentiating the *Enterobacteriaceae*; (8) the citrate test, which studies the organism's ability to apply citrate as a carbon source and is also helpful in differentiating the *Enterobacteriaceae*; (9) the nitrate reductase test, which investigates bacteria's ability or inability to reduce nitrate to nitrite; (10) the novobiocin sensitivity test, which evaluates an organism's suspectibility to novobiocin, an antibiotic that obstructs DNA

replication, and is helpful in differentiating among Gram-positive cocci; and (11) the diffusible pigment test using king agar, which differentiates among species of *Pseudomonas* by testing the secretion of pigments by the organism. Additionally, the bacteria isolates were viewed under a standard microscope to characterize their shape. The Omnilog Identification system was employed to further characterize the pool of environmental microorganisms.

The table in the Appendix depicts the results of the physical and biochemical tests carried out following Bergey's Manual of Determinative Bacteriology to establish the identify each microorganism. Using this methodology, all but nine of the isolates were identified to their genus. A total of 114 unique isolates were identified.

Next, the Omnilog Identification system (Omnilog) was used to confirm the genus identification of the isolates determined by the physical and biochemical tests and also to identify the species of the isolated organisms. The Omnilog was able to positively identify the genus of 54% of the isolates (61 of 114 isolates), with a few to the species level (see Appendix). The Omnilog was unable to identify any of the nine unknowns. The failure of the Omnilog system to identify the genus or species for a greater number of environmental isolates may be due to the limitations of the Omnilog database. The Omnilog database is relevant to primarily clinical microorganisms and not environmental microorganisms, like the bacterial isolates used in this study. In future efforts, an alternative method, such as genomic sequencing, would be more effective to confirm the genus identification results obtained from biochemical and physical analysis.

Table 2 summarizes the results of genus identification obtained through physical and biochemical analysis and Omnilog studies for each of the 114 unique environmental isolates collected. Excluding the unidentified organisms, seven unique genera were identified. Bacteria from the genus *Bacillus* and *Staphylococcus* were present in the greatest amount at 47% and 25% respectively. Conversely, only one isolate was identified from the genus *Aquaspirillum* and *Brevibacillus*. The greatest number of isolates were obtained from two of the AC ducts (57%) and the door handle (27%). Of the total number of isolates collected from the two AC ducts, 60% were identified as the genus *Bacillus*. These results are to be expected, as AC ducts contain a significant amount of debris on their interior surfaces and the spore forming bacteria *Bacillus* can easily become embedded there. Of the total number of isolates collected from the door handle, 84% were identified as the genus *Staphylococcus*. As *Staphylococcus* make up a significant portion of the skin microbiota, it is expected that the large quantity of *Staphylococcus* collected from the door handle is due to the frequent interaction of people's hands with the door handle.

Table 2. Summary of Genus Identification of Environmental Bacterial Isolates in Relation to Sample Collection Location

	Frequency								
	All Surfaces	Laundry	Kitchen sink	Cutting board	Tent 6 cot	Tent 6 AC	Tent 8 AC	Rigid shelt door handle	Rigid shelter AC
Organism Genus									
Aquaspirillum	1								1
Bacillus	53	4	7	1	1	2	23	1	14
Brevibacillus	1								1
Cupriavidus	11						11		
Micrococcus	7							2	5
Pseudomonas	3	1					2		
Staphylococcus	29	2						26	1
Unknown	9	2					3	2	2
Total	114	9	7	1	1	2	39	31	24

Characterization of Activity of Environmental Bacterial Isolates against the Target Bacteria

Selected isolates were screened for bacteriocin activity against three targets: *Bacillus anthracis* Sterne (surrogate for *Bacillus anthracis* from Colorado Serum Company), *Staphylococcus aureus* (ATCC 27217), or *Pseudomonas aeruginosa* (ATCC 15692) using soft agar overlays. A culture containing the target microorganism and a culture of the environmental isolate to be tested were inoculated into nutrient broth and incubated at 37 °C until an optical density (OD) of 1 (~10^8 cfu/mL) was achieved. Soft agar for overlay experiments was prepared with 7% agar in nutrient broth. Overlays were prepared by adding 60 µL of the target organism with 1.35 µL of a 100 mg/mL stock mitomycin C solution (Amresco Inc., Solon, OH) to a 7 mL aliquot of soft agar. The isolate was tested by dropping 6 µL of culture onto each plate in duplicate. The plates were incubated at 37 °C overnight. Activity was determined by the presence of a zone of clearing.

Table 3 shows a summary of the number of isolates that exhibited activity against the target strains evaluated. Since bacteriocins typically only demonstrate activity against close competitors, initial screens of bacteriocin activity against the target microorganisms focused on the same genus. *Bacillus* isolates and the nine unknowns were screened for activity against a *Bacillus anthracis* Sterne target; *Staphylococcu*s isolates and the nine unknowns were screened for activity against a *Staphylococcus aureus* target (ATCC 27217); and *Pseudomona*s isolates and the nine unknowns were screened for activity against a *Pseudomonas aeruginosa* target (ATCC 15692). None of the unknowns showed activity against any of the three targets. Of the 53 *Bacillus* isolates, 15% or 9 of the 62 isolates tested showed activity against the *Bacillus anthracis* Sterne target. Only one *Staphylococcus* isolate showed activity against the *Staphylococcus aureus* target. None of the *Pseudomonas* isolates exhibited activity against the *Pseudomonas aeruginosa* target. Secondary activity screens were performed to determine if any of the isolates produced bacteriocins against bacteria from the other target genera. All the isolates were screened against the *Staphylococcus aureus* and *Pseudomonas aeruginosa* targets. Only one of the isolates, a *Bacillus*, showed activity against the *Staphylococcus aureus* target,

while no isolates showed activity against the *Pseudomonas aeruginosa* target. Thus, only 2% or 2 out of the 114 isolates tested were found to be active against the *Staphylococcus aureus* target. Table 4 summarizes in greater detail the results of the isolates that were found to be active against the tested target strains.

Table 3. **Summary of Activity of Isolates Against Target Microorganisms**

Target Organism	Total isolates screened	Number of isolates positive for activity
Bacillus anthracis Sterne	62	9
Staphylococcus aureus	114	2
Pseudomonas aeruginosa	114	0

Table 4. **Detailed Summary of Active Isolates**

#	Name	Organism Identity	Target Active Against
62	FD6 b	*Bacillus subtilis*	BA
65	FD6 e	*Bacillus*	BA
142	FD8 o	*Bacillus*	BA
148	FD8 u	*Bacillus thuringensis*	BA
151	FD8 x	*Bacillus licheniformis*	BA
157	FD10 f	*Bacillus thuringensis*	BA
162	FD11 b	*Bacillus*	BA
164	FD11 d	*Bacillus*	BA
165	FD11 e	*Bacillus*	BA
60b	FD3 b	*Bacillus*	SA
105	FD7 g	*Staph*	SA

BA: *Bacillus anthracis* Sterne, SA: *Staphlocooccus aureus* (ATCC 27217)

The results of this work suggest that a low percentage of environmental isolates displayed bacteriocin-induced activity against the tested targets. The isolates were initially screened against a single target strain for each genera. In general, most bacteria produce bacteriocins against bacteria strains that exist as competitors in their environment, which contributes to the narrow activity spectrum of bacteriocins. Thus, a low number of isolates were anticipated to be active against the target bacteria strains. Within that context, the *Bacillus anthracis* bacteria isolated from environmental sources was expected to yield activity against the *Bacillus anthracis* Sterne target since *Bacillus anthracis* is a soil born bacteria. Nine isolates of *Bacillus* did in fact produce bacteriocins against the *Bacillus anthracis* Sterne target. Additionally, characterizing a single strain of *Staphylococcus* with activity against the target strain of *Staphylococcus aureus* was not surprising given that most of the *Staphylococcus* isolates were from the same door handle and may be the same species/strain. Most notably, one *Bacillus* isolate was discovered to elicit activity against the *Staphylococcus* target, yet did not demonstrate activity against the *Bacillus anthracis* Sterne target. Further, the isolate did not show activity toward any of the *Bacillus* strains tested. Further study of this isolate is warranted to understand why it is active against the *Staphylococcus aureus* target and not its *Bacillus* competitors.

The narrow-spectrum activity of the 11 environmental isolates that demonstrate bacteriocin-driven activity against the target strains was further confirmed with the assistance of Margaret Riley, a collaborator at the University of Massachusetts, Amherst. The 11 active isolates identified in the initial screening (Table 4) were tested for activity against additional targets of different genera and strains to characterize the activity spectrum exhibited by the isolates. The results of these additional activity screens showed that the 11 isolates exhibited no activity against unrelated genus strains and only minimal activity against varying strains of the same genus (results not shown). These findings reinforce that the bacteriocin-induced activity of the environmental isolates is specific and is only exhibited in a narrow-spectrum range. Thus, the nine isolates identified as active against *Bacillus anthracis* Sterne and the two isolates that demonstrated activity against *Staphylococcus aureus* resulting from this study are excellent candidates for development as narrow-spectrum antimicrobials to specifically target pathogens of interest to the DoD.

Summary of Task I

In summation of this task, to identify potential narrow-spectrum antimicrobials, a pool of environmental bacterial isolates was collected from several surfaces at Fort Devens and evaluated for their bacteriocin-driven activity against three target organisms of interest to the DoD. As expected, the greatest number of isolates were collected from surfaces that encountered the greatest use, which enabled a number of microorganisms to be transferred and/or to gather. All 114 environmental isolates collected were characterized. Not surprisingly, the common microorganisms of *Bacillus* and *Staphylococcus* comprised the majority of the environmental isolates at 47% and 25% respectively. Only nine of the *Bacillus* isolates demonstrated activity against the *Bacillus anthracis* Sterne target strain while one of the *Staphylococococcus* isolates demonstrated activity against the *Staphylococcus aureus* target strain. Interestingly, one other *Staphylococococcus* isolate exhibited activity against the *Bacillus anthracis* Sterne target. The low number of environmental isolates found to be active against the target strains evaluated illustrates the narrow-spectrum activity of bacteriocins. Moreover, screening of the active isolates against additional microorganisms revealed minimal activity, which further validates the specific bacteriocin-driven activity of the environmental isolates. The active environmental bacteria isolates provide a valuable resource for the development of narrow-spectrum antimicrobial agents. Future studies will build upon this work by isolating, purifying, and characterizing bacteriocins produced from these active environmental bacteria isolates.

Task II – Protocol for Initial Purification of Bacteriocins

The purpose of this task was to develop a protocol to instruct on the partial purification of an unknown bacteriocin to allow for rapid evaluation of its potential application as an antimicrobial against pathogenic strains of interest. Although bacteriocins have been studied for several decades, their utilization in industrial applications has been limited. Extensive time is required to produce, purify and characterize the activity of individual bacteriocins. Because the structure and molecular characteristics of bacteriocins are heterogeneous, a universal method for purifying sufficient quantities of bacteriocins for characterization is not readily available (Heng *et al*. 2007). A purification procedure must be developed for each unique bacteriocin, which may not necessarily translate to other bacteriocins of interest.

Traditionally, purification methods for bacteriocins developed in the laboratory are small-scale and include ammonium sulphate precipitation (Pingitore *et al*. 2007). While purification of bacteriocin is achieved using this process, ammonium sulphate precipitation is an expensive and labor intensive process when scaled-up to industrially required quantities. To overcome this limitation, development of a low-cost, universal purification protocol was sought, which would yield a bacteriocin sample pure enough to conduct preliminary activity screening. Employing tangential flow filtration in conjunction with ion exchange column chromatography was chosen as a practical foundation for developing the purification protocol. In the developed protocol described below, bacteriocins were isolated from cellular extracts using tangential flow filtration, which is similar to purification methods carried out in industry (personal conversation, Immucell, Portland, ME). Three filters with differing molecular weight cutoffs (MWCO) were used sequentially.

Development of Purification Protocol

Initially, procedures for purification of different bacteriocins published in the literature were evaluated (Herschman *et al* 1967). Comparison of the different schemes indicated several commonalities. An initial purification protocol was derived from the consensus, which included four steps: optimization of bacteriocin production from the producer, preparation of the cell lysate/extract, column purification, and desalting. To track the peptide throughout the purification process, aliquots from each step of the purification procedure were reserved and evaluated for antimicrobial activity using a soft agar overlay activity test. A zone of clearing was indicative of the presence of bacteriocin.

Multiple bacteriocin-producing bacteria species were assayed using the initial protocol to determine the efficacy of production of a purified peptide. The results showed that, though the peptide was cleaner than a crude lysate/extract, additional purification was required to assess its potential use in future studies. For example, **Figure 1** shows the results of the bacteriocin PA-18 purified using this purification procedure. PA-18 is a bacteriocin discovered from a clinical isolate of *Pseudomonas aeruginosa* and was found to be produced optimally in Brain Heart Fusion media. **Figure 1A** is a Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel. Lane 1 shows the protein marker and Lane 2 shows the crude lysate of PA-18. Lanes 3 and 4 are dilutions of the crude lysate while Lane 5 is the resulting lysate after purification using an ion exchange column. As seen in Figure 1A, the ion exchange column successfully eliminated some contaminating proteins (Lane 5) in comparison to the untreated crude lysate (Lane 2). The presence of PA-18 was confirmed by an activity overlay assay,

shown in **Figure 1B**. The zone of clearing present in Lane 5 of **Figure 1B** is indicative of the presence of PA-18. A zone of clearing is also present in Lane 2, signifying the presence of the antimicrobial peptide in the crude lysate. The results from this study suggest that additional purification steps are necessary to obtain a greater purity of the bacteriocin.

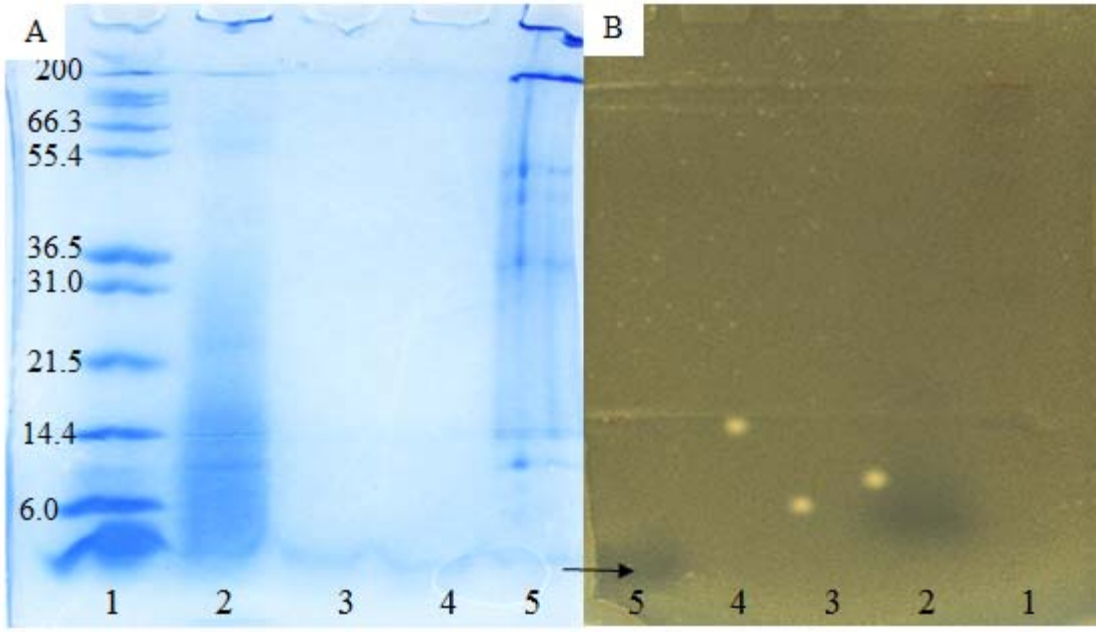


Figure 1. Purification of bacteriocin PA-18 using preliminary purification protocol. A) SDS-PAGE gel. B) Activity overlay assay.

To optimize the protocol an additional evaluation of the literature was conducted. From these findings the protocol was amended to include the addition of filtration before loading the peptide onto a column with the intention that more of the contaminating extracellular debris would be eliminated. An optimized protocol was designed to include tangential flow filtration. The lysate/filtrate of the bacteriocin producer was subjected to tangential flow filtration using filters with a 30,000 (30k) MWCO, 10,000 (10k) MWCO, and 1,000 (1k) MWCO pore size to remove unwanted protein/debris. After filtration, the resulting filtrate was purified further using the ion exchange column as detailed previously.

Application of the new protocol indicated the filters significantly increased the purity to a level sufficient to allow essential assessment of a bacteriocin's potential as an antimicrobial. **Figure 2** shows a representative SDS-PAGE gel employed to analyze the purity of PA-18 using the optimized protocol. Lane 1 shows the protein marker while Lanes 2 and 3 show purified nisin (used as a standard). Lane 4 was empty, though the observance of protein bands suggests that the neighboring lane (Lane 3) was overloaded and some of the sample migrated to Lane 4. Lane 5 shows PA-18 that was purified using tangential flow filtration, using all three aforementioned MWCO filters, followed by ion exchange chromatography. As seen in Lane 5, only one protein band is observed, suggesting that most contaminating proteins were removed using this amended procedure.

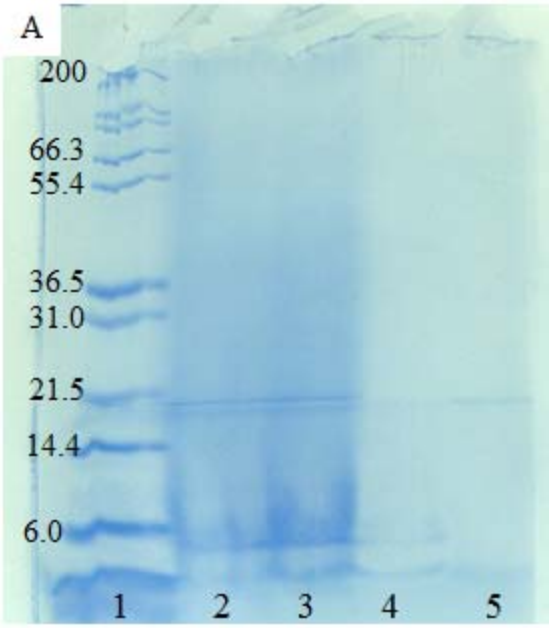


Figure 2. Purification of bacteriocin PA-18 using optimized purification protocol

Summary and Scope of Purification Protocol

Initially, this protocol evaluates the producer bacteria for optimal bacteriocin production in different media and under different conditions (i.e. temperature, presence of chemical inducer, etc.). Once the optimal conditions are determined, cell lysates/extracts are prepared and filtered through a 0.22-µm filter to remove cellular debris. The filtrate is further filtered through 30k, 10k and 1k MWCO filters using tangential flow filtration. Retentates and filtrates are tested for activity, and the active fraction(s) is (are) loaded onto an equilibrated Diethylaminoethyl (DEAE) Sephadex A-25 column. The peptide is eluted using 350 mM and 1 M Sodium Chloride (NaCl) in Tris buffer. The flow through, wash, and elution fractions are tested for activity. The active fraction is evaluated for purity, total peptide concentration, peptide size, peptide stability, and Minimum Inhibitory Concentration (MIC).

Activity at each stage is determined using the soft agar overlay method. Fields of the target organism are created by inoculating soft agar with a 4 h culture of the target bacteria and pouring the mixture over a media plate. Cell extracts or bacteriocin preparations are dropped onto the field and incubated overnight. The presence of active bacteriocin is determined by the observation of a zone of inhibition. If the activity is low or absent, samples are concentrated using a SpeedVac and then re-tested for activity.

Purity of the peptides is determined using a Bis-Tris PAGE gel. The peptide band is identified using a soft agar overlay of the gel. Peptide concentration is determined using the Bicinchoninic Acid (BCA) protein assay. The MIC assay performed was the microtiter variation with inhibition assessed on a spectrophotometer, measuring OD at 600 nm. Stability is determined by incubating aliquots of the peptide at different temperatures and testing for retained activity at different times using the soft agar overlay method.

Apparatus, Reagents and Materials Required for Purification Protocol

- Incubator
- Sterilizer
- Shaker incubator
- Agar plates – Use a combination of Nutrient, Tryptic Soy, Brain Heart Infusion (BHI), Mueller Hinton II, and Lysogeny Broth (LB) plates. Prepare each as directed.
- Broth – As with the plates, prepare broth as directed for use with liquid cultures.
- Centrifuge – Run at 10,000 rpm for 30 min to remove cells and cellular debris from cell lysates/extracts.
- Tangential flow – Process used to filter the samples. Includes washing the retentates with 25mM Tris buffer pH 8.5. Wash the filter with MQ-water and 0.2 mM of Sodium Hydroxide (NaOH), and then store it in 0.1 N Phosphoric acid.
- Tris buffer – Prepare 1 L of 25 mM Tris-HCl. Bring to pH 8.5 using NaOH and Hydrochloric acid (HCl). When needed for column elution, add NaCl to the buffer to increase the overall salt molarity.
- DEAE Sephadex – Equilibrate column using the 25mM Tris buffer. After elution, wash column with 2M NaCl and 0.1M NaOH as needed.
- Bis-Tris PAGE gel – Prepare using either (1) 40% acrylamide, Tetramethylethylenediamine (TEMED), SDS and ammonium persulfate as a 10-12% gel or (2) purchased pre-poured as a 4-12% gel. Prepare samples with sample buffer and load as 20-µL quantities into the wells.
- SimplyBlue Coomassie stain – Purchased from Invitrogen. Prepare gels as described in procedures provided by the vendor.
- Eon Microplate Spectrophotometer – used for MIC and BCA assay.
- BCA reagent
- Biological Safety Cabinet – Required when using Class II or higher microorganisms.

Preparation of Target Organisms

Three strains of bacteria, chosen for their relevance to Army interests, were used as model pathogens to evaluate the activity of the purified bacteriocins. The three target bacteria were: *Bacillus anthracis* Sterne (Colorado Serum Company), *Staphylococcus aureus* ATCC 27217, and *Pseudomonas aeruginosa CD81*. They were stored at -80 ^{o}C until needed.

The procedure for preparing a culture of organisms is as follows:

- Inoculate a single colony of a fresh culture of *Bacillus anthracis Sterne, Staphylococcus aureus,* or *Pseudomonas aeruginosa* from a streak plate into 10 mL of culture broth recommended for each target strain.
- Incubate the culture on a shaker incubator set at 37 °C for 4 h, or until the OD measures 1 AU at 600 nm.
- To prepare a soft agar overlay activity assay, add 40 µL of this culture to 7 mL of soft agar, and pour mixture over a media plate.

Preparation of Bacteriocin Sample for Purification or Optimal Media Screening

Bacterial isolates from clinical and/or environmental sources were stored as glycerol stocks at -80 ^{o}C until needed.

To produce bacteriocin samples:

- Inoculate a single colony of a fresh culture of the test isolate from a streak plate into 10 mL of broth culture.
- Incubate the culture on a shaker incubator at 37 °C for 4 h or until an OD of 1 is achieved.
- Inoculate a 1 L flask of broth with this culture if running a purification test, or inoculate plates with the culture for use with the optimal media test.

Determination of Optimal Media for Bacteriocin Production

To generate the greatest yield of bacteriocin, several media were evaluated for their effects on bacteriocin production.

To determine the optimal media that generates the most bacteriocin:

- Prepare rectangular media plates*:*
  - Fill each plate with 30 mL of sterile media.
  - Inoculate 12 mL of soft agar with 20-40 µL of the prepared target organism culture and pour it over the media plate. To evaluate the effect of mitomycin C on production of bacteriocin, use plates with and without the addition of mitomycin C. If using mitomycin C, add 1.6 µL of the mitomycin stock to the soft agar before pouring onto the media. The composition of the soft agar and media should be the same.
  - Allow the soft agar to harden.
- Test the samples*:*
  - For each isolate, drop 6 µL of the 10 mL culture used in preparing the target organism onto the plate. Test duplicates at two different locations on each plate to confirm results for each media type and test multiple isolates on a single plate.
  - Incubate each plate at either 30 or 37 °C overnight to determine optimal temperature. Evaluate activity by the presence of a zone of inhibition and by growth of the isolate.

Purification of Bacteriocin

The following steps were established to yield purified bacteriocin:

- Incubate the 1 L culture sample prepared for purification at the optimum temperature as determined in the previous section, with agitation, for 16 h.
- Remove the cells via centrifugation and filter the supernatant through a 0.22-µm filter.
- Filter and test the filtrate:
  - Filter the filtrate through a 30k MWCO filter using tangential flow filtration and wash the retentate twice with 200 mL of Tris buffer (which, at this point, should reduce the filtrate to about 150 mL).

    - Filter the filtrate through a 10k MWCO filter and again wash the retentate twice with Tris buffer.
    - Filter the filtrate through a 1k MWCO filter and again wash the retentate twice with Tris buffer.
    - Test all filtrates and retentates for activity, both in an un-concentrated and concentrated form.
- Perform an MIC on the semi-purified peptide using the microtiter plate procedure to determine activity.
- Test the fractions:
    - Load the most filtered fractions demonstrating activity onto the DEAE Sephadex column, and collect the flow through.
    - Wash the column with 200 mL of Tris buffer and collect the eluted fractions.
    - Elute the peptide using a step gradient with Tris buffer containing 150, 250, and 350 mM or 1M NaCl.
    - Test each fraction for activity, both in an un-concentrated and concentrated form.
- Analyze the active sample for total protein concentration using the BCA assay.

Determination of Bacteriocin Purity

The following steps were established to determine the purity of produced bacteriocin:

- Load 20 µL samples of the active fractions onto Bis-Tris PAGE. As a general note, samples with 150-mM and greater NaCl concentrations must be desalted using dialysis or G10 Sephadex columns against Tris buffer to prevent interference when the gel is run.
- Stain the gels with SimplyBlue Coomassie stain and destain them in water.
- To identify the protein band representing the bacteriocin, use an agar overlay activity assay to determine which protein band demonstrates activity.
    - Wash the gel in water to remove SDS and place it onto a Petri dish over media.
    - Inoculate soft agar with the target organism, pour it over the gel, and incubate overnight.
    - Compare pictures of the stained and overlayed gel to identify the active peptide band as determined by a zone of inhibition

Determination of Stability of Purified Bacteriocin

Evaluation of the bacteriocin after purification is necessary to ensure that the activity of the bacteriocin is retained.

The following method was followed to evaluate the stability of the purified bacteriocin:

- Incubate aliquots of crude or purified material at different conditions (temperature, pH, time, etc.) for a given period of time.
- Perform activity tests to determine stability.

Summary of Task II

In Task II, a universal purification protocol was developed to purify bacteriocins from the cell lysate or extract of a producer organism. A universal purification protocol is necessary to

facilitate the rapid assessment of the antimicrobial peptides for activity against pathogenic organisms of interest. Currently, the heterogeneous nature of bacteriocins as well as the myriad methods employed in the lab to purify the antimicrobial peptides renders their purification to be uniquely specific for each bacteriocin investigated (Carolissen-Mackay *et al*. 1997). Additionally, the small-scale purification of bacteriocins in the lab often consists of ammonium sulphate which is cost-prohibitive for large-scale industrial processing (Pingitore *et al*. 2007). The universal purification protocol developed in this task overcomes these limitations.

Tangential flow filtration followed by ion exchange chromatography was found to provide sufficient purity of bacteriocins as determined by SDS-PAGE. As a result, these techniques comprised the universal purification protocol. Further evaluation of the protocol is necessary to confirm validity. For instance, a more sensitive separation technique such as High Performance Liquid Chromatography (HPLC) would yield additional information on the resulting purity of the antimicrobial peptides. Additionally, quantifying the yield of bacteriocin obtained using this protocol would be necessary for comprehensive evaluation of the universal purification protocol. If the yield of antimicrobial peptide obtained is too low to carry out numerous assays to screen for activity, alternative techniques may be assessed to generate more peptide. Optimized yield of an individual bacteriocin may be achieved by building upon the universal purification protocol established in this task and amending the method as necessary.

Task III – Development of Purification Protocol Specific for Bacteriocin 105b

Developing a bacteriocin for applications to protect the Warfighter from pathogenic bacteria requires a bacteriocin that demonstrates strong activity against an Army relevant pathogenic microorganism and can be consistently produced in high quantities and purities. The purpose of this task was to establish a specific purification protocol for an isolated bacteriocin of interest. The bacteriocin of focus in this task was bacteriocin sample 105b isolated from Fort Devens in the initial collection of the bacterial pool for screening of activity against target pathogens of interest to the Army in Task I. Bacteriocin 105b exhibited potent, targeted activity against *Bacillus anthracis* Sterne, a surrogate strain of *Bacillus anthracis*, the etiological agent of anthrax. Preliminary studies utilizing bacteriocin 105b have shown the antimicrobial agent to be produced constitutively with strong yields. Because of this, bacteriocin 105b demonstrates promise for application into platforms such as textiles, material coatings or wipes to impart antimicrobial functionality and protect the Warfighter from pathogenic bacteria. To conduct further studies aimed at achieving these results, a purification protocol was produced specific to bacteriocin 105b to provide high yields and sufficient purity of the antimicrobial agent.

Development of Purification Protocol Specific for Bacteriocin 105b

Previous work detailed in Task II established a generic purification protocol to apply to any bacteriocin to procure yields and purities sufficient for future tests to evaluate the activity of the antimicrobial agents. The procedure developed in Task II focused on developing a universal purification protocol that could yield sufficiently pure material to facilitate the rapid evaluation of the bacteriocin's activity against target pathogenic microorganisms. In this method, bacteriocins were isolated from cellular extracts by using tangential flow filtration. Three filters with differing MWCO were used sequentially. To further eliminate contaminants, ion exchange column chromatography was used. This purification method provided the foundation to tailor the procedure specifically for bacteriocin 105b. The results obtained in Task II were carefully analyzed to determine areas that could be targeted for improvement to increase the yield of bacteriocin 105b. For example, it was observed that the fraction collected from tangential flow filtration that exhibited the greatest activity was the retentate collected from the 30k MWCO filter with minimal activity observed in the resulting filtrate obtained from the 1k MWCO filter. This result provided insight into the purification of bacteriocin 105b and provided the launching point for the investigation carried out in this task.

Apparatus, Reagents and Materials Required for Purification of Bacteriocin 105b

- Incubator
- Sterilizer
- Incubator shaker
- Nutrient agar plates
- Tryptic Soy Broth – Prepare as directed for use with liquid cultures.
- Centrifuge – Perform centrifugation at 10,000 RPM for 30 min to remove cells and cellular debris from cellular extracts.
- Tangential flow – Subject the samples to tangential flow filtration with a 30k MWCO filter. Wash retentates using 25mM Tris buffer pH 8.5. Wash the filter with MQ-water, 0.2 mM NaOH, and then store it in 0.1 N Phosphoric acid.

- Tris Buffer – Prepare 25mM Tris-HCl and bring to pH 8.5 using NaOH and/or HCl. When needed for column elution, add NaCl to the buffer to increase the overall salt molarity.
- DEAE Sephadex – Equilibrate columns using the 25mM Tris buffer. After elution, wash the column with 2M NaCl, and 0.1M NaOH as needed
- Bis-Tris PAGE gel – Prepare using either 40% acrylamide, TEMED, SDS and ammonium persulfate as a 10-12% gel, or purchased pre-poured as a 4-12% gel from Invitrogen. Prepare samples with sample buffer and load as 20-µL quantities into the wells.
- Simplyblue Coomassie stain – Purchased from Invitrogen. Prepare and stain gels as described in vendor procedure.
- Eon and Omnilog microtiter plate reader – used for MIC and BCA assay.
- BCA reagent
- Biological Safety Cabinet – Required when using Class II or higher microorganisms

Preparation of Target Organism

Bacteriocin 105b demonstrates strong activity against *Bacillus anthracis* Sterne (from Colorado Serum Company), a surrogate for the live, active toxic agent producing strain of *Bacillus anthracis*. *Bacillus anthracis* Sterne was stored at -80 °C until needed. A fresh culture of *Bacillus anthracis* Sterne was prepared by inoculating a single colony from a streak plate into 10 mL nutrient broth. The culture was incubated on a shaker incubator at 37 °C for 4 h at 220 rpm, or until the OD measured 1 AU at 600 nm. To prepare soft agar overlays for activity tests, 40 µL from this culture was added to 7 mL of soft agar, which was then poured over tryptic soy broth (TSB) media and allowed to harden.

Production of Bacteriocin 105b

A fresh culture of the bacteriocin 105b producer strain *Bacillus subtilis* was prepared by inoculating a single colony from a streak plate into 10 mL of TSB. The culture was incubated on a shaker incubator at 37 °C for 4 h, or until the OD measured 1 AU at 600 nm. A 1 L flask of TSB was inoculated and incubated with agitation for 16 h at 37 °C.

Purification of Bacteriocin 105b

The development of a universal purification protocol to purify bacteriocins ("Protocol for Initial Purification of Bacteriocin", 2015), sequentially implemented 30k, 10k and 1k MWCO filters for tangential flow filtration, where the filtrate of the preceding filter was applied as the flow through for the next filter. Bacteriocin 105b was initially purified from its host using the universal protocol. During this study, the molecular weight of bacteriocin 105b was determined to be around 4 kilodaltons (kDa) using SDS-PAGE. Due to its molecular weight, bacteriocin 105b was predicted to be isolated in the retentate of the 1k MWCO filter. However, the resulting retentate collected from the 1k filter provided a poor yield of bacteriocin 105b. To increase the yield of bacteriocin 105b achieved through purification, a purification protocol specific to 105b was developed, building upon the findings of the previously employed universal purification method.

To isolate bacteriocin 105b from the cellular extract, the culture in the previous section was first centrifuged at 10,000 rpm for 30 min to separate the cells. The supernantant was filtered through a 0.22 µm filter. Tangential flow filtration was then used to filter the resulting filtrate. The purification of bacteriocin 105b through the process of tangential flow filtration using three different MWCO filters was evaluated to determine where the antimicrobial peptide is retained.

The filtrate from the 0.22 µm filter was subject to tangential flow filtration using a 30k, 10k and 1k MWCO filter. The retentate for each filter was washed twice with 600 mL of 25 mM Tris buffer at pH 8.5. A 1 mL aliquot of both the retentate and filtrate were concentrated using a SpeedVac to 1/10th the volume (100 µL). Aliquots for the retentate and filtrate of each filter were collected and activity tests were conducted at each filter step to assess the presence of bacteriocin 105b.

Results of the activity test indicated bacteriocin 105b is retained in the retentate of the 30k MWCO filter. **Figure 3** shows the activity of the respective retentates and filtrates for each filter size. As seen in **Figure 3**, zones of clearing indicative of activity induced from the presence of bacteriocin 105b are only present in the crude sample (no tangential flow filtration, panel A) as well as the retentate remaining from tangential flow filtration over the 30k MWCO filter (panel B). This result indicates that bacteriocin 105b does not pass through the 30k MWCO filter and affirms that additional processing using smaller MWCO filters in tangential flow filtration is not necessary to purify bacteriocin 105b. As a result, the utilization of 10k and 1k MWCO filters for tangential flow filtration was eliminated from the purification process specific for bacteriocin 105b. Also of note, the small colonies observed in the zone of clearing in the aliquot of the 30k retentate suggest the presence of contaminating bacteria resistant to bacteriocin 105b. This serves as a reminder that clean laboratory techniques must be executed to ensure contaminating microorganisms are restrained.

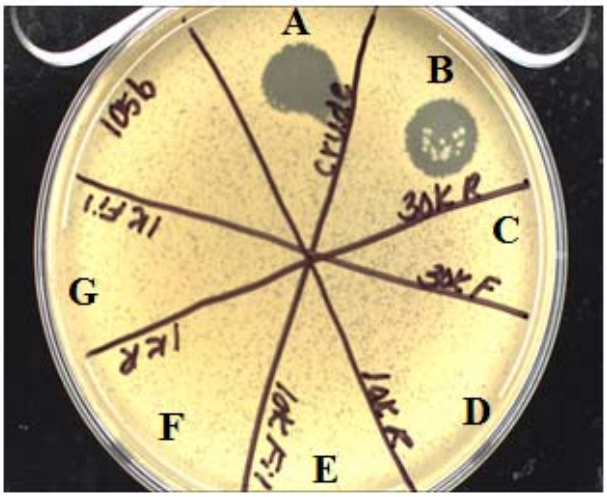


Figure 3. Isolation of bacteriocin 105b using tangential flow filtration. A) Crude bacteriocin 105b sample not subjected to tangential flow filtration. B) Retentate from 30k MWCO filter. C) Filtrate from 30k MWCO filter. D) Retentate from 10k MWCO filter. E) Filtrate from 10k MWCO filter. F) Retentate from 1k MWCO filter. G) Filtrate from 1k MWCO filter.

Because the proposed size of bacteriocin 105b is much smaller than the expected size range for retention using the 30k MWCO filter, this result was unanticipated. Many conditions associated with the tangential flow filtration process may affect the retention of a protein. One hypothesis that may explain why bacteriocin 105b does not flow through the 30k MWCO filter is that the antimicrobial peptide interacts with other solutes such as polyglutamic acid, a biopolymer also excreted by *Bacillus subtillus*. Due to this interaction, bacteriocin 105b may not exist as a free peptide in solution, which may account for the antimicrobial activity observed in the retentate of the 30k MWCO tangential flow filtration filter. If bacteriocin 105b does not remain a free

peptide in solution, this justification may also suggest that molecular interactions are necessary for bacteriocin 105b to retain its stability when extracted from the cell. The nature of the proposed interaction between polyglutamic acid and/or other proteins in the cellular extract is not known. Additional studies are required to fully comprehend the interaction of bacteriocin 105b with other excreted molecules and their effects on the stability and activity of bacteriocin 105b. Polyacylamide gel electrophoresis in conjunction with an activity overlay assay would evaluate the size of the potential protein complex containing bacteriocin 105b. Additional assays to dissociate the complex and characterize the associated proteins would also be necessary.

Once bacteriocin 105b was isolated from the cellular extract using tangential flow filtration, additional steps were performed to remove remaining contaminants. The retentate was then passed through a DEAE Sephadex A-25 (GE Healthcare) column equilibrated with 25 mM Tris buffer pH 8.5 to further eliminate contaminating proteins from bacteriocin 105b. The column was washed with 25 mM Tris buffer pH 8.5 containing 150 mM or 1M NaCl to ensure any potential bacteriocin 105b was removed from the column. The collected fractions were assessed for activity by activity drop test and evaluated for purity using PAGE to visualize the number of protein bands. **Figure 4** shows a representative activity drop test that evaluates the activity of fractions collected from DEAE Sephadex column chromatography to isolate the presence of bacteriocin 105b. **Figure 4** shows zones of clearing only for the 30k MWCO filter retentate that was applied to the ion exchange column chromatography and the aliquot collected of the resulting flow through. Activity is not observed after washing the column with 150 mM NaCl in 25 mM Tris buffer at pH 8.5. This result suggests that bacteriocin 105b does not interact with the DEAE Sephadex column. Instead, the antimicrobial peptide passes through the column and is eluted in the flow through. Again, contaminating bacteria colonies resistant to bacteriocin 105b are observed in the zones of clearing observed in **Figure 4**.

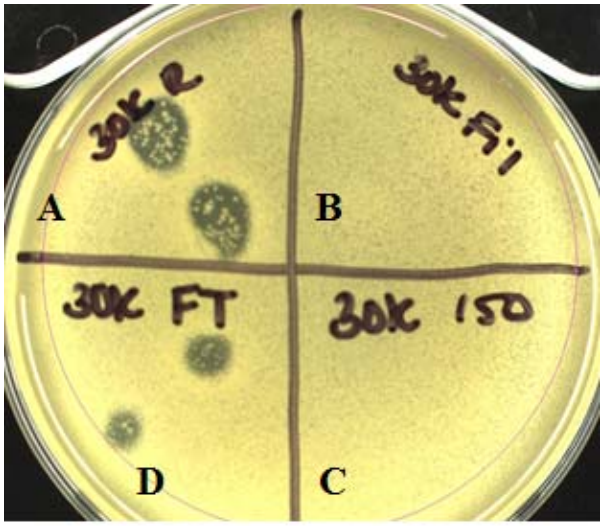


Figure 4. Isolation of bacteriocin 105b from purification with ion exchange column chromatography Dropped samples: A) 30k MWCO filter retentate. B) 30k MWCO filter filtrate. C) Flow through after applying 30k MWCO retentate to ion exchange column. D) Wash fraction collected after washing column with 25 mM Tris buffer 150 mM NaCl.

Evaluation of Protein Purity

To evaluate the purity and query the yield of bacteriocin 105b collected from the DEAE Sephadex fractions, gel electrophoresis was used. Bis-Tris PAGE gels were prepared either using 40% acrylamide, TEMED, SDS and ammonium persulfate as a 10-12% gel or gels were purchased pre-poured as 4-12% gels from Invitrogen. Fractions resulting from 150 mM and 1M NaCl washes were desalted to prevent interference when run on the gel. The fractions were desalted using dialysis or G10 Sephadex columns against 25 mM Tris buffer pH 8.5. Samples were run in duplicate lanes on the same gel. At the end of electrophoresis the gel was cut in half. One half of the gel was stained using SimplyBlue Coomassie stain (Life Technologies) to visualize the protein bands. The other half of the gel was assessed for activity. The gel was overlayed onto media and a soft agar overlay inoculated with the target microorganism was poured over the gel and incubated overnight at 37 °C. Pictures of the stained and overlayed gels were compared to identify the active peptide band represented by a zone of clearing in the target overlay.

PAGE was carried out to assess the purity of bacteriocin 105b in the flow through collected after retentate from the 30k MWCO filter was applied to an ion exchange column. **Figure 5** shows a representative PAGE gel with samples collected from ion exchange column chromatography. Lane 1 shows the Novex Sharp Pre-Stained Protein Standard. Lane 2 shows the flow through collected after passing the 30k MWCO retentate over the column. Lanes 3 and 4 show the fractions collected after washing the column with 250 mM NaCl and 1 M NaCl, respectively. As seen in **Figure 5** the greatest amount of protein is present in the fraction collected after washing the column with 250 mM NaCl (Lane 3). Substantially less protein is observed in the flow-through collected from ion exchange column chromatography, where activity tests indicate bacteriocin 105b is retained (Lane 1). This result suggests that most of the extraneous protein in the 30k retentate interacts with the column and is only eluted after a salt wash. This result also shows minimal contaminants were present in the fraction of the flow through collected (Lane 2), where antimicrobial activity was observed when the fraction was evaluated for activity (Figure 2).

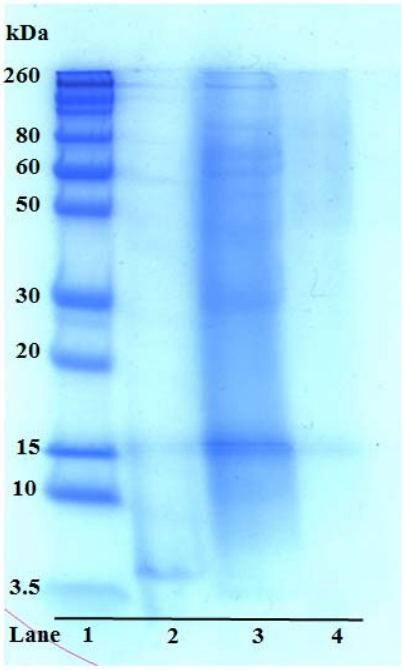


Figure 5. Purification of bacteriocin 105b after ion exchange column chromatography

To confirm the presence of bacteriocin 105b in the flow through fraction analyzed by PAGE, a gel analyzing the flow through collected from ion exchange column chromatography was assessed for antimicrobial activity. **Figure 6A** shows a representative PAGE gel with the flow through collected after applying the 30k retentate to the DEAE Sephadex column (Lanes 1 and 2). Only one protein band is observed around 4 kDa. **Figure 6B** shows the results of an activity overlay assay of the gel. Two zones of clearing are observed which relate to the two protein bands observed in **Figure 6A**. This result suggests that the protein band at 4 kDa is bacteriocin 105b which was eluted from the column during the flow through. The presence of contaminating proteins in the flow through fraction is not observed, which may suggest the purity of bacteriocin 105b is high. However, contaminating proteins may be present in the purified 105b sample but may not be visible by the PAGE analysis due to their low abundance. To confirm the absence of contaminating proteins, a higher concentration of the purified 105b sample could be evaluated by PAGE and/or characterized using a more sensitive visualization technique, such as silver staining. The purified 105b sample could also be characterized by Fast Protein Liquid Chromatography (FPLC) to determine proteins present.

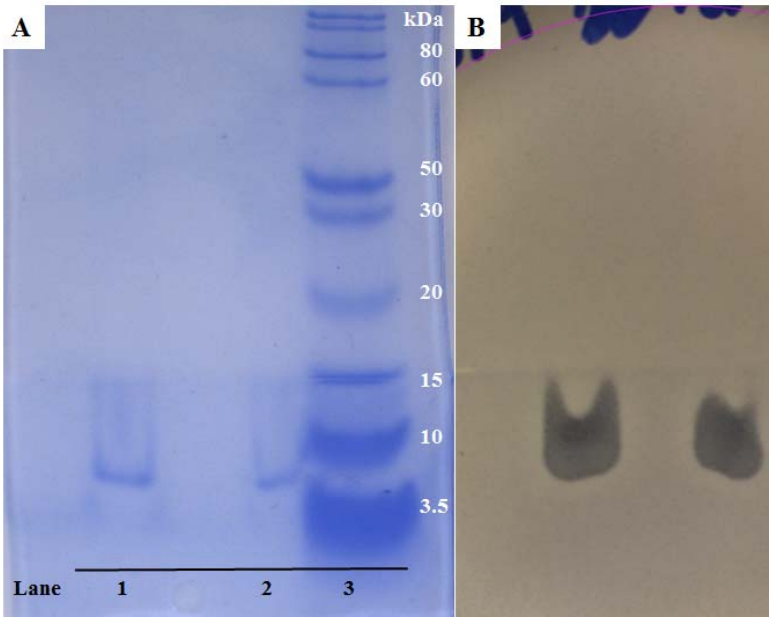


Figure 6. Confirmation of presence of bacteriocin 105b after ion exchange chromatography. A) SDS-PAGE gel stained with Coomassie Blue. B) Results of activity overlay of duplicate gel pictured in Figure 4A.

To comprehensively evaluate the developed purification protocol specific for bacteriocin 105b, the quantified yield of bacteriocin 105b must be considered. Ideally, the developed purification protocol should generate a high quantity of the antimicrobial peptide at a high purity. Work is currently ongoing to elucidate the quantity of bacteriocin 105b that is produced using this method. The BCA assay will be applied to assess the total protein concentration obtained in the sample of purified 105b. Because the crude extract from the bacteriocin 105b producing host is subjected to less processing using tangential flow filtration than in the universal purification protocol, it is expected that the total protein concentration will be greater. Liquid chromatography methods such as FPLC and/or HPLC will be utilized to isolate bacteriocin 105b and characterize its concentration to determine the yield of antimicrobial peptide obtained.

Summary of Task III

In order to progress with employing bacteriocin 105b in applications designed to impart antimicrobial functionality, a purification procedure was tailored to produce a high purity of bacteriocin 105b. Previous work developing a universal purification protocol for quickly characterizing bacteriocins was leveraged as the foundation for this work. It was similar to purification methods employed by industry (Immucell, Portland, ME). This purification method employed tangential flow filtration to rapidly yield a crude bacteriocin sample with enough purity to evaluate activity. This process is advantageous to purification procedures developed in the laboratory, which predominantly employ precipitation by ammonium sulfate (Pingetore *et al*. 2007) and do not readily translate to large-scale industrial applications due to high cost and labor. As detailed in this report, building upon the universal purification protocol, a purification protocol specific to bacteriocin 105b was achieved. Tangential flow filtration in conjunction with anion exchange column chromatography was employed. Initial characterization of the ensuing sample of antimicrobial peptide suggests a high yield of bacteriocin 105b was acquired. Further evaluation is necessary to confirm the purity and establish the quantity produced of

bacteriocin 105b. The heterogeneous nature of bacteriocins as well as the numerous and varying protocols employed to purify bacteriocins makes it difficult to compare the results of this purification protocol to others (Carolissen-Mackay *et al*. 1997). Furthermore, bacteriocin 105b is an environmental isolate whose identity is currently unknown.

In future studies regarding bacteriocin 105b, the antimicrobial peptide should be sequenced to ascertain if the antimicrobial peptide is novel or has been previously characterized in literature. It is envisioned that bacteriocin 105b will be used in a textile that demonstrates antimicrobial activity to protect the Warfighter from pathogenic bacterial threats. To accomplish this goal, the stability of bacteriocin 105b in a textile must be evaluated and supplemented as necessary to ensure the antimicrobial peptide remains active. Long-term stability and activity of bacteriocin 105b should be established to determine the effects of potential storage conditions of the antimicrobial textile in an employable environment. Additionally, the activity of bacteriocin 105b against commensal flora, as well as the identity of any remaining impurities in the extract of bacteriocin 105b, must be evaluated to ensure the safety of the Warfighter. The development of a purification protocol specific to bacteriocin 105b to yield a highly pure antimicrobial peptide with narrow-spectrum activity is a significant step towards advancing a novel construct to provide antimicrobial protection to the Warfighter.

Task IV – Entrapment of Bacteriocin 105b onto Fabric with Titania

The purpose of this task was to conduct an initial evaluation of the process of fabricating a multifunctional textile by incorporating antimicrobial and photocatalytic functionalities into a single textile through the encapsulation of a narrow-spectrum antimicrobial, bacteriocin 105b, by precipitation with titania, a photocatalytic matrix, on a swatch of fabric. The resulting material is anticipated to exhibit both antimicrobial and photocatalytic properties.

Conventionally, fabrication of multifunctional protection for the Warfighter entails the development of separate components that each address an individual issue. A multifunctional platform is then achieved upon combination and integration of the individual components to form a single system that displays more than one property. While the current process for developing a multifunctional system does facilitate multiple threats to be addressed by one scheme, the resulting system can be clumsy and restricting. The individual elements that comprise a multifunctional system, each focused on eliminating a singular threat, may not be readily modular or may not function in the conditions necessary for another component upon integration. To overcome this challenge, the BSTT has been investigating novel, non-traditional textiles to advance the development of multifunctional materials for incorporation in protective garments. The goal is to prepare materials with multiple functionalities developed in unison to augment the protection of the Warfighter, while simultaneously diminishing the cost of production and the weight of the uniform. One strategy being examined to accomplish this aim is the encapsulation of biologics, such as bacteriocins, in a metal oxide matrix on fabric to simultaneously impart multiple functionality to the textile.

In this task, preliminary studies were conducted to investigate the encapsulation of bacteriocin 105b with the chosen encapsulation matrix of titanium dioxide, also known as titania. Titania exhibits photocatalytic properties, making it an excellent option for the foundation of a multifunctional bionanocomposite (Gaya *et al*. 2008). Previous studies have elucidated a facile method to precipitate titania under mild, biologically friendly conditions using biomimetic synthesis (Filocamo *et al*. 2011 and Luckarift *et al*. 2006). In these studies, an enzyme was successfully encapsulated in titania and demonstrated to retain its activity. In addition, work by the BSTT (Filocamo) through the FORCE ProTex project has demonstrated the encapsulation of the bacteriocin nisin using titania precipitation. To investigate the preparation of a multifunctional textile with a different antimicrobial spectrum, the findings of the FORCE ProTex efforts were leveraged and the feasibility of encapsulating an alternative bacteriocin, 105b, in the titania matrix on fabric to yield a multifunctional textile was evaluated. The work described in this task builds upon the findings of the FORCE ProTex effort by employing a similar methodology using bacteriocin 105b and evaluates the feasibility of encapsulating an alternative bacteriocin with a different narrow-spectrum range of antimicrobial activity.

Preparation of Bacteriocins for Entrapment in Titania

Two bacteriocins were used in this study: nisin and the 105b isolate.

Preparation of Nisin: The nisin stock solution was prepared by measuring out 0.125 g of 20% (w/v) nisin and adding it to 25 mL of 25 mM Tris buffer pH 7.5 to give a final concentration of 1 mg/mL of nisin. The nisin stock solution was vortexed for 5 min and spun down at 3000 rpm for 10 min. The supernatant was retained for future use.

Preparation of 105b: The 105b isolate was prepared for use as a semi-pure preparation and a pure preparation. To prepare the semi-pure preparation, a 10 mL nutrient culture was inoculated with the 105b isolate and grown for 4 h. This culture was used to inoculate a 1 L culture with 30 g tryptone, 5 g sucrose, 7 g dipotassium phosphate, 2 g monopotassium phosphate, 0.5 g sodium citrate, 0.1 g magnesium sulfate and 1 g ammonium sulfate. The culture was grown overnight at 37 °C. The cells were removed via centrifugation and the supernatant was retained. The supernatant was filtered through a 30,000 MWCO filter using tangential flow. The retentate was kept and used as the semi-pure preparation of 105b. To prepare the pure preparation of 105b, the retentate was further processed over a DEAE Sephadex A-25 column washed with 25 mM Tris buffer pH 8.5. As the 105b bacteriocin does not stick to the column, the pure peptide sample was collected in the flow through and subsequent washes of the column. To investigate the effect of pH on the activity of bacteriocins, the pH of pure 105b was adjusted using hydrochloric acid to pH 4.5, 5.5 and 6.5 and then immediately evaluated for activity.

Activity of Bacteriocins Entrapped in Titania in Solution

Before investigating the activity of bacteriocins after entrapment in titania in solution, the antimicrobial activity of solutions of nisin, semi-pure 105b and pure 105b was confirmed using the soft agar overlay method. **Figure 7A** illustrates the zones of clearing observed for each bacteriocin against *Bacillus anthracis* Sterne. As expected, all the bacteriocin solutions tested exhibited activity, with nisin and pure 105b exhibiting the greatest zones of clearing. Though the semi-pure 105b did exhibit a zone of clearing, it was visibly smaller than the zones observed for nisin and pure 105b, suggesting that the semi-pure 105b exhibits less activity than pure 105b and nisin. This finding was expected, as the semi-pure sample of 105b was subject to less purification. This resulted in a smaller concentration of 105b in the protein content of the semi-pure 105b sample in comparison to the pure preparation of the 105b sample whose protein content is mostly the 105b peptide.

To determine the effect of pH on the activity of 105b, the pH of pure 105b was adjusted to 4.5, 5.5 and 6.5 and then immediately evaluated for activity. As seen in **Figure 7A**, the pure preparation of 105b at each pH tested exhibited activity as inferred from the observation of zones of clearing. This observation indicates that the activity of pure 105b is retained over the range of pH's tested. Additionally, the solution of 105b at various pH's was stored at 4 °C for several weeks before again being evaluated for activity. Activity was still observed even under these conditions (results not shown) indicating that the pure 105 remains stable and active when stored at the pH range tested.

Before precipitation with titania, the pH of bacteriocin solutions was adjusted to pH 6.5 with the addition of hydrochloric acid. To precipitate titania with bacteriocin in solution, 24.5 mL of bacteriocin solution was combined with 0.5 mL of titanium (IV) bis-(ammonium lactate)-dihydroxide (TBALD) pH 6.5. Precipitation occurred on a shaker at 250 rpm for 6 h. Titania precipitated bacteriocin solutions were then evaluated for activity without further purification.

The activity of bacteriocins encapsulated in titania in solution was evaluated by the soft agar overlay method against *Bacillus anthracis* Sterne. **Figure 7B** depicts the results.

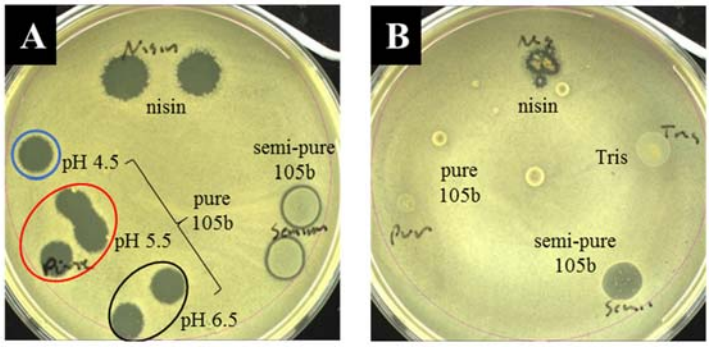


Figure 7. Activity of bacteriocins in solution. A) Without encapsulation in titania. Samples tested were nisin, semi-pure 105b and pure 105b at pH 6.5 (black circle), 5.5 (red circle) and 4.5 (blue circle). The colored circles have been added to assist with visualization. B) Activity of titania encapsulated bacteriocins in solution after titania precipitation. Samples tested were nisin, Tris buffer (negative control), semi-pure 105b and pure 105b.

As expected, titania precipitated in the absence of a bacteriocin did not yield a zone of clearing, indicating that the titania matrix is not antimicrobially active. This finding confirms that titania is a suitable encapsulation medium for the bacteriocins. The precipitated titania with nisin exhibited a zone of clearing, indicating that the activity of nisin is retained when entrapped. This finding is consistent with results of the FORCE ProTex effort. Although semi-pure 105b appears to exhibit less activity than pure 105b in solution (**Figure 7A**), when precipitated with titania, the encapsulated semi-pure 105b retained activity while pure 105b did not. The difference in activity observed for the encapsulated 105b samples may be due to the presence of contaminating elements and/or additional proteins in the semi-pure preparation of 105b. These elements may act as molecular stabilizers which protect the peptide from degradation during the precipitation process, preserving the activity of the peptide. Additionally, the lack of activity observed for the encapsulated pure 105b material may be due to the absence of these stabilizers, which may have been stripped away during the purification process, promoting the inactivity of the pure peptide. Also of particular note, the semi-pure 105b appears to exhibit greater activity when encapsulated in titania than when not encapsulated. Several hypotheses may explain this observation. The titania precipitate may disrupt potential aggregation of the peptide in solution, resulting in more uninhibited, active peptide to be encapsulated. The precipitation of titania may draw the bacteriocins from solution to encapsulate a high concentration of bacteriocin, resulting in the observation of greater activity. Additional studies are required in order to investigate and ascertain the mechanism responsible for the increased activity of semi-pure 105b when encapsulated in precipitated titania.

Activity of Bacteriocins Entrapped in Titania on Fabric Swatches

Swatches of NyCo, a 50/50 nylon cotton blend currently used to produce Army Combat Uniforms (ACUs) were used as the representative fabric of interest for testing. To prepare the NyCo for the entrapped bacteriocin in titania, the swatches were scoured in borax by incubating at 80 °C for 1 h. The swatches were then washed with water and air dried. Scoured swatches to be precipitated with titania were pre-treated by being submerged in 2.5% Reputex^{TM} for 45 min. The swatches were removed from the Reputex^{TM} and placed between six paper towels. The samples were “squeezed” between the paper towels to remove excess water. To bond the Reputex^{TM} to the fabric, the samples were heated to 120 ^{o}C for 10 min.

To investigate the development of a multifunctional textile, NyCo swatches with titania encapsulated bacteriocins were tested for antimicrobial activity using soft agar overlays against *Bacillus anthracis* Sterne. The presence of activity was noted by a zone of clearing observed around the swatch. Since Reputex™, the precipitation inducing agent, exhibits antimicrobial activity, swatches only treated with Reputex™ were first evaluated to determine the extent titania precipitation could cover the surface and eclipse the antimicrobial activity of Reputex™. **Figure 8** shows the results of this first study. All the swatches evaluated were tested for activity immediately following precipitation (**Figure 8A**, 0 h) and after incubation for 24 h at 4 °C (**Figure 8B**, 24 h). Untreated swatches (UT) of NyCo did not exhibit any activity verifying that the NyCo fabric is not antimicrobial. Swatches treated with Reputex™ without titania precipitation (R) exhibited a zone of clearing around the perimeter of the swatch signifying activity, while swatches treated with Reputex™ in the presence of titania precipitation (R+Ti) did not. This confirms that the precipitation of titania successfully masked the antimicrobial activity of the Reputex™ on the treated swatches at both time points tested.

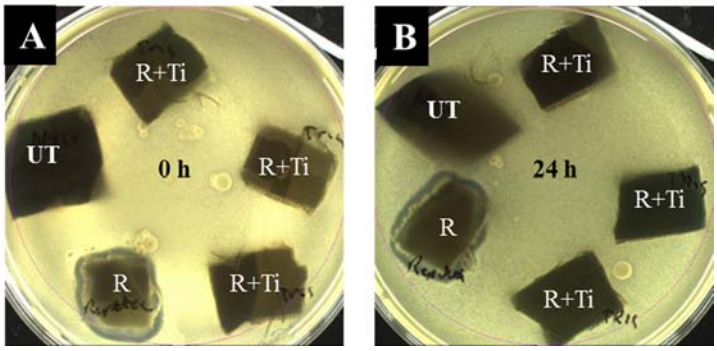


Figure 8. Activity of control swatches with and without the presence of titania. A) Right after treatment (0 h). B) After the treated swatches were incubated for 24 h at 4 °C

The activity of bacteriocins entrapped by titania precipitation on Reputex™ treated NyCo swatches was evaluated using the soft agar overlay method against *Bacillus anthracis* Sterne. As seen in **Figure 9**, swatches with nisin encapsulated by titania exhibited a zone of clearing around the swatch indicating that the bacteriocin remains active following precipitation with titania. The nisin encapsulated swatches retain their activity even after incubation at 4 °C for 24 h. These findings are in agreement with previous studies evaluating activity of swatches coated with titania encapsulated nisin completed by the FORCE ProTex effort. The activity of semi-pure 105b and pure 105b encapsulated by titania precipitation on Reputex™ treated swatches was also investigated. **Figure 10** shows the activity of swatches with titania encapsulated semi-pure 105b, while **Figure 11** shows the activity of swatches with titania encapsulated pure 105b. As seen in **Figure 10**, after immediate testing and after storage at 4 °C for 24 h, one of the three swatches with titania encapsulated semi-pure 105b demonstrated activity as evidenced by the zone of clearing around the swatch highlighted by the added arrows in the figure. No activity was observed for swatches encapsulated with pure 105b as seen in **Figure 11**. These results are in agreement with the observations for titania encapsulated semi-pure and pure 105b in solution (**Figure 7B**), where only the semi-pure 105b encapsulated in titania demonstrated activity. As with the precipitation of titania in the presence of the bacteriocins in solution, the difference in activity observed for the semi-pure 105b and pure 105b preparations is hypothesized to be due to the difference in the preparation conditions for the two samples. The remaining impurities in the semi-pure preparation

of 105b may act as a stabilizer that keeps the peptide active. As these impurities are removed during further purification to obtain pure 105b preparation, the peptide may become unstable, resulting in a loss of activity. This has been seen with other peptides and proteins. Further testing is necessary to confirm this hypothesis and investigate alternative conditions to promote the antimicrobial activity of titania encapsulated purified 105b on swatches.

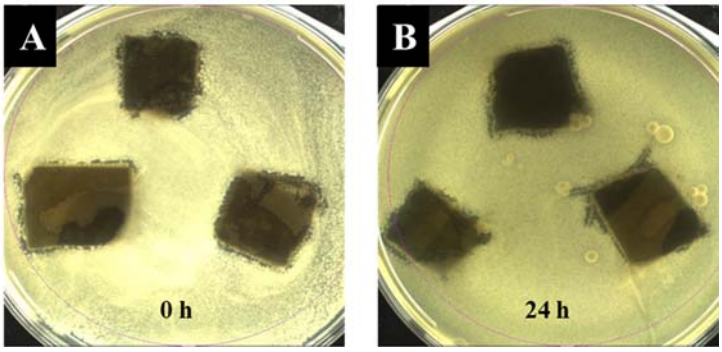
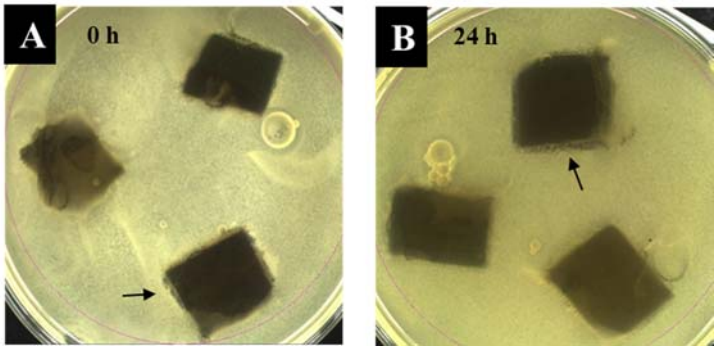


Figure **9**. Activity of nisin entrapped in titania on fabric. A) Right after treatment (0 h). B) After the treated swatches were incubated for 24 h at 4 °C



*Note: arrows have been added to point out the observed zones of clearing around the swatches

Figure 10. Activity of semi-pure bacteriocin 105b entrapped in titania on fabric. A) Right after treatment (0 h). B) After the treated swatches were incubated for 24 h at 4 °C

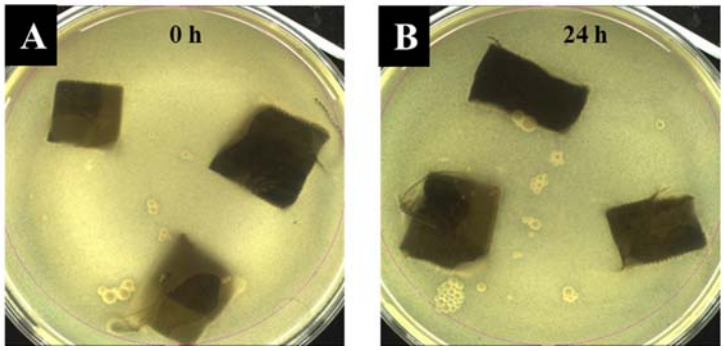


Figure 11. Activity of pure bacteriocin 105b entrapped in titania on fabric. A) Right after treatment (0 h). B) After the treated swatches were incubated for 24 h at 4 °C

Summary of Task IV

Preliminary investigations concerning the preparation of a multifunctional textile were conducted. The antimicrobial activity of bacteriocin 105b entrapped in titania in solution and on NyCo fabric swatches was investigated. Two preparations of 105b were studied, a semi-pure preparation and a further purified preparation. The results from these studies demonstrate that in solution, the pure preparation of 105b is more active than the semi-pure preparation of 105b. When encapsulated in titania in solution or on fabric, the semi-pure preparation of 105b retains its activity while the pure preparation of 105b does not.

From these findings, it is hypothesized that the experimental conditions employed to purify 105b are detrimental to the stability of the peptide. This is most likely due to the loss of molecular stabilizers during the purification process which are necessary for the peptide to retain its activity. Future work by evaluating the addition of molecular stabilizers may improve the stability of the pure peptide. Additional studies are also required to further investigate the preparation of a multifunctional material by encapsulation of an antimicrobial agent by titania. Examples of these studies include, but are not limited to, confirming the catalytic activity of the titania nanoparticles once precipitated in the presence of bacteriocin as well as addressing the need to control the release and activity of the bacteriocin to ensure optimal use.

Task V – Optimization of Bacteriocin 105b Production in Defined Media

A high yield of bacteriocin 105b is required to employ the peptide as an antimicrobial agent in applications designed to protect the Warfighter from pathogenic bacteria in the field. In industry, production of a bacteriocin reaches yields of up to 100 mg/L (personal conversation, Immucell, Portland, ME). The purpose of this task was to evaluate growth conditions, while taking into account cost, to optimize the production of bacteriocin 105b from its *Bacillus subtilis* host, isolated in Task I. This work was carried out from June 2014-September 2015.

Although a moderate quantity of bacteriocin 105b was acquired using the purification protocol developed in Task III, optimization of the production of bacteriocin 105b from its host is necessary to increase the quantity of the peptide. Evaluation of the literature suggests the production of bacteriocins is affected by growth conditions including temperature and pH (Drosinos *et al*. 2005). Additionally, the source of carbon and nitrogen present in the growth media has been found to significantly influence the production of bacteriocins (Mahrous *et al*. 2013, Mataragas *et al*. 2004). Initially, several complex media formulations were evaluated to determine their effects on the yield of bacteriocin 105b produced. Based on the results of this study, the effects of carbon and nitrogen sources present in the growth media were evaluated for effects on the production of bacteriocin 105b. The production of the antimicrobial peptide was assessed by monitoring activity, with the expectation that the magnitude of activity observed correlates to the magnitude of quantity produced.

Recently, several studies have focused on the utilization of low-cost, ecologically friendly growth media components in the production of bacteriocins. These economically viable production strategies significantly reduce the cost to produce bacteriocins on a large scale, which is necessary in industrial or future applications using the antimicrobial peptide to impart protection to the Warfighter. Production methodologies for bacteriocins have been developed using media containing cheese whey, soybean residue (Leaes *et al.* 2011), mussel-processing waste (Perez Guerra *et al*. 2002) as well as byproducts from the sugar industry including low-cost sugars and molasses (Metsoviti *et al*. 2011). These unique, low-cost media formulations provide an alternative to conventional laboratory preparations of bacteriocins.

In this task, the optimization of bacteriocin 105b production from the producer organism discovered in Task I (*Bacillus subtilis* 105b) was evaluated using several media formulations. First, the yield of bacteriocin 105b was assayed in several conventional complex media formulations. Next, several carbon and nitrogen sources were investigated for their effects on bacteriocin 105b production. Finally, molasses media was studied as an alternative media formulation for the production of bacteriocin 105b. The ease of availability and low cost of molasses media makes it an ideal solution for bacteriocin production.

Comparison of Complex Media on Bacteriocin 105b Production

The yield of bacteriocin 105b was investigated upon growth of the host organism in several common complex media. The media selected for testing were: Lysogeny Broth (LB, from Becton, Dickinson and Co.), Nutrient Broth (NB, from Himedia), TSB (from Becton, Dickinson and Co.), Mueller-Hinton II (MHII, from Becton, Dickinson and Co.), and BHI (from Becton, Dickinson and Co.). Additionally, a combination of equal volumes MHII and BHI was also

studied for effects on bacteriocin 105b production. To grow the 105b producer organism, a single colony of the bacteriocin producer strain, *Bacillus subtilis* strain 105b, was inoculated in 10 mL of MHII. This was stored in a shaker incubator at 37 °C at 220 rpm for 4 h or until an OD of 1 was achieved. A 100 µL aliquot of this culture was transferred into 10 mL of each media to be evaluated in triplicate and incubated for 16 h in an incubator shaker at 37 °C at 220 rpm.

To assess the production of bacteriocin 105b in each test media, a time kill-kinetic assay was performed. For each media evaluated, bacteriocin 105b was prepared by removing a 1.5 mL aliquot from the overnight culture described above. The aliquot was centrifuged and the supernatant was lyophilized. Samples were re-suspended in sterile water such that the total volume was equal to 250 µL.

The target organism for the MIC assay was *Bacillus anthracis* Sterne. The target was prepared by growing it in nutrient broth until an OD of 1 was achieved. The culture was diluted by 1:100 in nutrient broth.

The time kill-kinetic assay was carried out in a 96-well microtiter plate. The concentrated bacteriocin 105b cell extract was serially diluted in nutrient broth such that 50 µL of the bacteriocin 105b cell extract was in each well at 100%, 50% and 25% respectively. To each well was added 150 µL of target. The positive control consisted of 150 µL of target with 50 µL of each complex media. The negative control was 200 µL of nutrient broth. An Eon™ High Performance Microplate Spectrophotometer (Biotek) was used to analyze the optical density of the kinetic assay over time. The optical density of the target organism is expected to vary based on the quantity of bacteriocin 105b present.

Figure 12 shows the results of the time kill-kinetic assay to evaluate the production of bacteriocin 105b in different complex media. The x-axis represents the six different complex media tested while the y-axis shows the OD at 600 nm after 18.5 h of incubation. Each concentration of bacteriocin 105b producer cell extract tested is represented by a different color. As expected, the growth of the target organism was greatest when no bacteriocin was present. The greatest inhibition of growth against the target was observed for bacteriocin 105b produced in TSB. Minimal growth of the target was observed for all three concentrations of cell extract tested. This finding suggests that high antimicrobial activity is present which suggests that a high quantity of bacteriocin 105b is produced when the host organism is grown in TSB. Conversely, the other media investigated yielded high optical densities during the time kill-kinetic assay which suggests that production of bacteriocin 105b from the host organism in these media is low. As a result of this experiment, TSB was deemed to be the superior media for optimized production of bacteriocin 105b. Further, an activity drop test assay was performed with each cell extract on an overlay of target. Results from this experiment confirm the findings from the kinetic assay (results not shown).

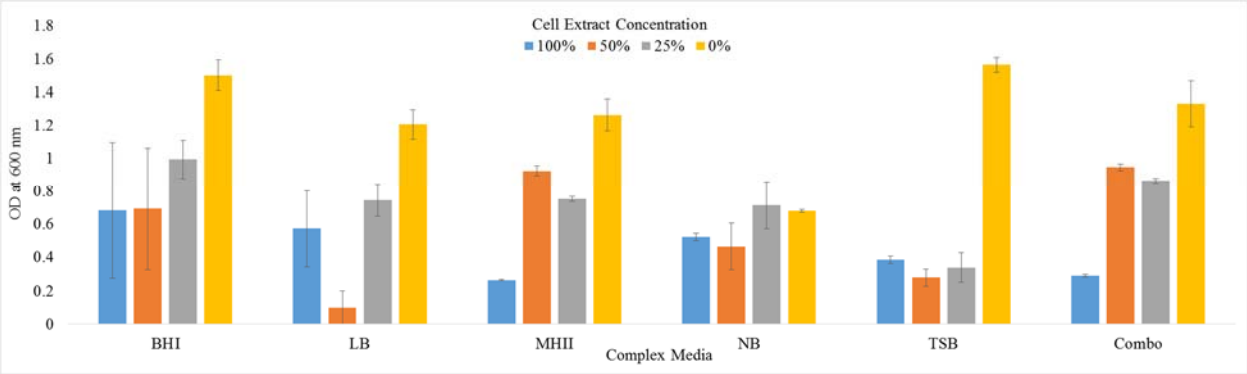


Figure 12. Results of time kill-kinetic assay to investigate the production of bacteriocin 105b in complex media. The complex media evaluated were: Brain Heart Infusion (BHI), Lysogeny Broth (LB), Mueller-Hinton II (MHII), Nutrient Broth (NB), Tryptic Soy Broth (TSB) and a 50:50 combination of BHI and MHII (combo). Serially diluted bacteriocin 105b cell extract serially diluted to yield 100%, 50% and 25% of concentration were combined with the target organism *Bacillus anthracis* Sterne. Using a spectrophotometer, the OD of each sample was analyzed over time. High values of OD indicate low activity of bacteriocin 105b, suggesting a low quantity of bacteriocin 105b present, which may indicate that the production of bacteriocin 105b in the tested media was low.

Effect of Nitrogen Source on Production of Bateriocin 105b

As previously stated, the source as well as the concentration of nitrogen in the growth media can influence the rate of production for a bacteriocin from its host organism (Mataragas *et al*. 2004). According to the manufacturer's (Becton, Dickinson and Co.) product brochure, the nitrogen sources in TSB consist of 17 g tryptone and 3 g soytone. To further optimize the production of bacteriocin 105b to yield the highest quantity possible, several nitrogen sources at different concentrations were evaluated in minimal media. As with the comparison of complex media, a time kill-kinetic assay was used to evaluate the effects of nitrogen source and concentration on the generation of bacteriocin 105b.

Five nitrogen sources were investigated. They were tryptone (Fisher Scientific), peptone (Sigma Aldrich), sodium nitrate ($NaNO_3$, Sigma Aldrich), ammonium nitrate (NH_4NO_3, Sigma Aldrich) and urea (Sigma Aldrich). The concentrations of each nitrogen source were varied to evaluate concentration effects on the production of bacteriocin 105b. The range of concentrations tested were chosen based around traditional concentrations of nitrogen sources in complex media as well as literature (Mohammed Abdel-Mawgoud *et al*, 2008). The concentrations tested for each nitrogen source are in **Table 5**. Each nitrogen formulation was added to minimal media supplemented with dextrose to prepare the growth media for the bacteriocin 105b host organism. Minimal media was prepared using the following formulation for 1 L: 7 g of dipotassium phosphate, 2 g monopotassium phosphate, 0.5 g sodium citrate, 0.1 g magnesium sulfate and 1 g ammonium sulfate.

Table 5. Nitrogen Sources and Concentrations Used to Evaluate Effects on Production of Bacteriocin 105b

Tryptone	Peptone	$NaNO_3$	NH_4NO_3	Urea
22 g/L	15 g/L	10 g/L	4.5 g/L	25 g/L
17 g/L	10 g/L	7.5 g/L	2.5 g/L	20 g/L
12 g/L	5 g/L	5 g/L	0.5 g/L	15 g/L

The bacteriocin producer organism, *Bacillus subtilis* strain 105b, was prepared by inoculating a single colony in 10 mL of TSB, which was incubated in a shaker incubator at 37 °C at 220 rpm for 4 h or until an OD of 1 was achieved. A 100 µL aliquot of this culture was transferred into 10 mL of each media to be evaluated in triplicate and incubated for 16 h in an incubator shaker at 37 °C at 220 rpm. A 1.5 mL aliquot from the overnight culture was centrifuged and the supernatant was lyophilized. Samples were re-suspended in sterile water such that the total volume was equal to 250 µL.

The target organism for the MIC assay was *Bacillus anthracis* Sterne. The target was prepared by growing it in nutrient broth until an OD of 1 was achieved. The culture was diluted by 1:100 in nutrient broth.

The time kill-kinetic assay was carried out in a 96-well microtiter plate. The concentrated bacteriocin 105b producer cell extract was serially diluted such that 50 µL of the bacteriocin 105b cell extract was in each well at 100%, 50% and 25% respectively. To each well was added 150 µL of target. The positive control consisted of 150 µL of target with 50 µL of nutrient broth. The negative control was 200 µL of nutrient broth. An Eon™ High Performance Microplate Spectrophotometer (Biotek) was used to analyze the optical density of the kinetic assay over time. The optical density of the target organism is expected to vary based on the quantity of bacteriocin 105b present, which is dependent on the production of the antimicrobial peptide in the media compositions tested.

Figure 13 shows the results of the investigation of six nitrogen sources at various concentrations and the relationship on the generation of bacteriocin 105b. The x-axis shows the nitrogen sources evaluated as well as the chosen concentrations tested. The y-axis shows the OD at 600 nm after 17 h. Each dilution of the bacteriocin 105b producer cell extract is represented by a different color. The yellow bar (0%) represents the OD of growth of the target in the absence of any bacteriocin 105b producer cell extract (positive control). A change in OD in the presence of bacteriocin 105b producer cell extract compared to the positive control is indicative of the activity of the antimicrobial peptide which may correlate with the quantity present, where the magnitude of change is suggestive of the quantity of bacteriocin 105b. It can be inferred that the greater the difference in OD (i.e. the lower the OD value) the greater the activity of the bacteriocin, suggesting a greater yield of bacteriocin 105b for the respective nitrogen source evaluated. The results in **Figure 13** show that sodium nitrate and ammonium nitrate performed as the worse sources of nitrogen for the production of bacteriocin 105b as their cell extracts at all concentrations exhibited the least inhibition on the growth of the target. This finding suggests that both nitrate salts are not sufficient sources of nitrogen in the production of bacteriocin 105b. Conversely, both tryptone and peptone exhibited the greatest inhibition on the growth of the target, suggesting that a high yield of bacteriocin 105b was produced when the antimicrobial peptide producer was grown in media containing these nitrogen sources. As TSB, the complex media discovered to yield the greatest production of bacteriocin 105b, contains tryptone, this result is not surprising. The findings of this study confirm that the resulting peptones of peptide digestion (peptone and tryptone) are optimal nitrogen sources for the production of bacteriocin 105b. Additionally, an activity drop test assay was performed with each cell extract on an overlay of target and results from this experiment confirm the findings from the kinetic assay (results not shown).

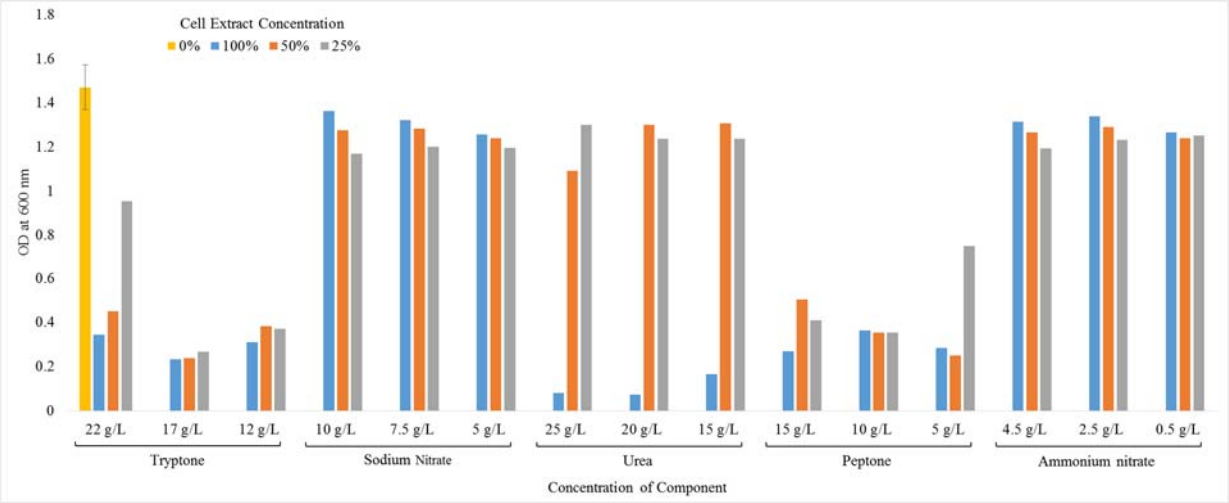


Figure 13. Results for the evaluation of different nitrogen sources and concentrations on production of bacteriocin 105b. The x-axis represents the six different nitrogen sources employed (tryptone, sodium nitrate, urea, peptone and ammonium nitrate) at different concentrations. The y-axis represents OD values at 600 nm. Results for the different dilutions of bacteriocin 105 producer cell extract are represented with different colors.

Effect of Carbon Source on the Production of Bacteriocin 105b

Just as the source of nitrogen in the growth media can affect the production of bacteriocins, so does the source of carbon available in the media (Mataragas *et al*. 2004). Thus, evaluation of carbon sources as they relate to the production of bacteriocin 105b is necessary to identify the source of carbon that yields an optimized quantity of the antimicrobial peptide. For reference, in the manufacturer's (Becton, Dickinson and Co.) product brochure for TSB, the optimal complex media investigated in the first section of this task, the carbon source is listed as 2.5 g dextrose. The screening of carbon sources carried out in this section was based on literature (Mohammed Abdel-Mawgoud *et al*, 2008), where the optimal production of a biosurfactant was found to be 2 g/L. Concentrations of carbon sources both above and below this value were investigated. To further optimize the production of bacteriocin 105b to yield the highest quantity possible, several carbon sources at different concentrations were evaluated in TSB devoid of dextrose. As in the previous comparison studies, a time kill-kinetic assay was used to evaluate the effects of carbon source and concentration on the generation of bacteriocin 105b.

The eight carbon sources chosen to evaluate the production of bacteriocin 105b were dextrose, fructooligosaccharides, galactooligosaccharides, glucomannan, mannose, molasses, D-sorbitol and surcrose. All carbon sources were purchased from Sigma-Aldrich. Each sugar was evaluated at three concentrations: 3 g/L, 2 g/L, and 1 g/L. The carbon sources were combined with TSB without dextrose. TSB was chosen due to previous findings which suggested that bacteriocin 105b production was optimal in TSB.

The bacteriocin producer organism, *Bacillus subtilis* strain 105b, was prepared by inoculating a single colony in 10 mL of TSB, which was incubated in a shaker incubator at 37 °C at 220 rpm for 4 h or until an OD of 1 was achieved. A 100 µL aliquot of this culture was transferred into 10 mL of each media with unique carbon sources to be evaluated in triplicate and incubated for 16 h in an incubator shaker at 37 °C at 220 rpm. A 1.5 mL aliquot from the overnight culture was centrifuged and the supernatant was lyophilized. Samples were re-suspended in sterile water such that the total volume was equal to 250 µL.

The target organism for the MIC assay was *Bacillus anthracis* Sterne. The target was prepared by growing in nutrient broth until an OD of 1 was achieved. The culture was diluted by 100 in nutrient broth.

The time kill-kinetic assay was carried out in a 96-well microtiter plate. The concentrated bacteriocin 105b producer cell extract was serially diluted such that 50 µL of the bacteriocin 105b cell extract was in each well at 100%, 50% and 25% respectively. To each well was added 150 µL of target. The positive control consisted of 150 µL of target with 50 µL of nutrient broth. The negative control was 200 µL of nutrient broth. An Eon™ High Performance Microplate Spectrophotometer (Biotek) was used to analyze the optical density of the kinetic assay over time. The optical density of the target organism is expected to vary based on the quantity of bacteriocin 105b present, which is dependent on the production of the antimicrobial peptide in the media compositions tested.

Figure 14 shows the results of the evaluation of carbon sources added to TSB absent of dextrose on the production of bacteriocin 105b using the kill time-kinetic assay. The x-axis shows the different carbon sources evaluated as well as the concentration range tested. The y-axis represents the OD at 600 nm for each sample. Dilutions of the cell extract of the bacteriocin producer are represented by the different color bars. A diminished OD suggests antimicrobial activity is observed due to the presence of bacteriocin 105b inhibiting the growth of the target. A lower OD implies greater inhibition of target growth, suggestive of a greater quantity of bacteriocin 105b produced. The results in **Figure 14** show varying antimicrobial activity such that no pattern of activity of bacteriocin 150b production is discernable. In order to conclusively evaluate the results of this study, the time kill-kinetic assay should be repeated at least two more times in order to assess more accurate and reproducible data. It is expected that repeating the experiment may yield more fruitful results and that the data may show a discernable correlation between the source of carbon present in the growth media and the quantity of bacteriocin 105b produced.

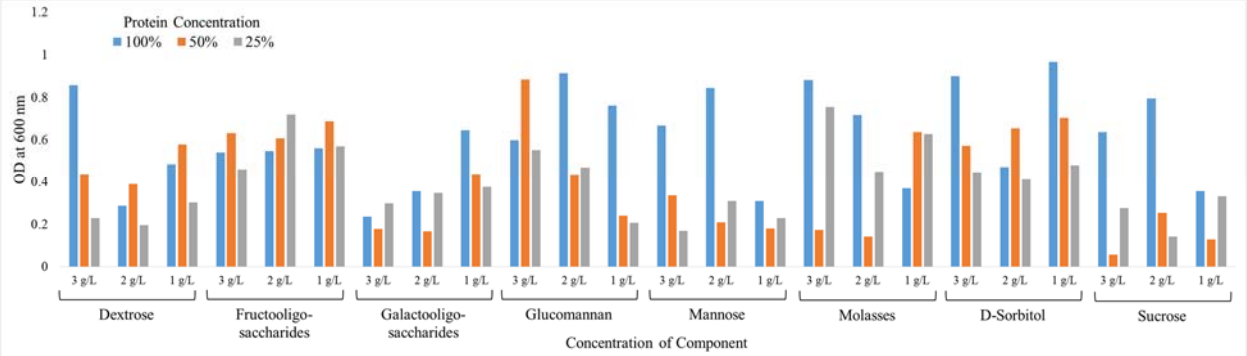


Figure 14. Results of time kill-kinetic assay to screen carbon sources for the determination of optimal production of bacteriocin 105b. Eight carbon sources were evaluated at concentrations of 3 g/L, 2g/L and 1 g/L. The x-axis shows the carbon source and the concentration while the y-axis shows the OD at 600 nm after 10 h. Dilutions of bacteriocin 105b producer cell extract are represented by the different color bars.

In parallel, an activity drop test was performed using the undiluted cell extract of bacteriocin 105b producer for each carbon source investigated against an overlay of the target organism, *Bacillus anthracis* Sterne. **Figure 15** shows the results of this assay. Each panel in **Figure 15** shows a picture of an activity drop test. **Figure 15A** shows the results for cell extracts with dextrose, D-sorbitol and fructooligosaccharide. **Figure 15B** shows results for cell extracts gown in the presence of mannose, molasses and sucrose. **Figure 15C** shows results for cell extracts

grown in the presence of fructoolisaccharide, galactooligosaccharide, glucomannan and cell extract grown in TSB without the addition of a carbon source. The result for cell extract grown in the presence of sucrose at 1 g/L is shown in **Figure 15D**.

Zones of clearing, indicative of the antimicrobial activity arising from the presence of bacteriocin 105b, can be observed for several cell extracts. A greater zone of clearing observed implies a greater antimicrobial activity exhibited due to a greater quantity of bacteriocin 105b present. The observation of "spots" within the zone of clearing may be due to the growth of bacterial colonies of the bacteriocin 105b producer organism as the cell extract was not filtered. In order to conclusively evaluate the effects of the producer organism on antimicrobial activity to ensure its presence does not contribute to the observation of activity, the assay should be repeated with filtered lysate. Importantly, no zone of clearing was observed for the cell extract collected from the producer organism grown in media devoid of a carbon source confirming the necessity of a carbon source for the production of the antimicrobial peptide (**Figure 15C**). Comparison of the strengths of the zones of clearing suggests that when dextrose, mannose, molasses and sucrose are used as carbon sources in the growth media for *Bacillus subtilis* 105b, a greater yield of bacteriocin 105b is achieved, as opposed to applying D-sorbitol, galacooligosaccharides, fructooligosaccharides and glucomannan as carbon sources. As with the kinetic assay, the drop test activity should be repeated to confirm the results observed during this initial study. Evaluation of the effects of carbon source on the generation of bacteriocin 105b preliminarily indicates that the source of carbon may be relevant to the yield of antimicrobial peptide produced with dextrose, mannose, molasses and sucrose, contributing to a greater yield.

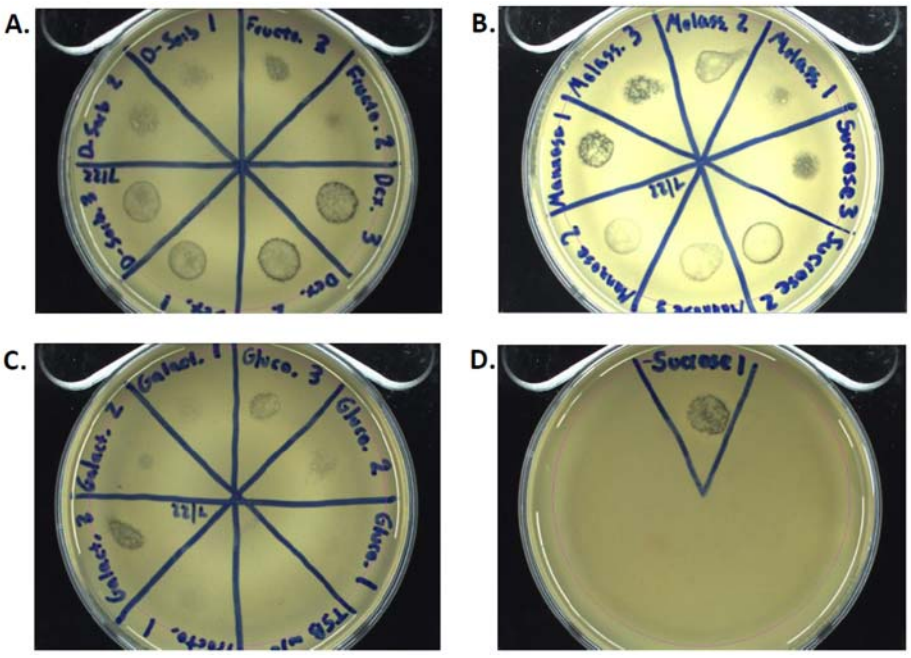


Figure 15. Results of activity drop test of cell extracts prepared from the bacteriocin 105b producer organism grown in TSB without dextrose and supplemented with alternative carbon sources. A) Cell extracts with dextrose (dex., 3 g/L, 2 g/L, 1 g/L), D-sorbitol (D-sorb., 3 g/L, 2 g/L, 1 g/L) and fructooligosaccharide (fructo., 3 g/L and 2g/L). B) Cell extracts gown in the presence of mannose (3 g/L, 2 g/L, 1 g/L), molasses (molass., 3 g/L, 2 g/L, 1 g/L) and sucrose (3 g/L and 2 g/L). C) Cell extracts grown in the presence of **fructooligosaccharide** (1 g/L), galactooligosaccharide (galact., 3 g/L, 2 g/L, 1 g/L), glucomannan (gluco., 3 g/L, 2 g/L, 1 g/L) and cell extract grown in TSB without the addition of a carbon source (TSB w/o). D) Cell extract grown in the presence of sucrose at 1 g/L.

Optimization of Bacteriocin 105b Production in Molasses Media

The targeted antimicrobial activity demonstrated by bacteriocin 105b against an Army relevant pathogenic organism alludes to the potential of this antimicrobial peptide to be employed in platforms to protect the Warfighter from biological threat. Large-scale, industrial production of bacteriocin 105b will be necessary to yield a suitable quality of the material for development and application. Thus, the production process of the antimicrobial peptide must not only be optimized to efficiently yield enough material but also be cost effective.

A cost analysis was first conducted to assess the expense associated with several growth media for the bacteriocin 105b producer, *Bacillus subtilis* 105b. The growth media considered were TSB, a composition of tryptone/sucrose/minimal media and a composition of molasses/trace minerals/minimal media. The growth media and components chosen for analysis were based on previous results acquired in the evaluation of complex growth media and its components on the effects of bacteriocin 105b production as described previously. In the screen of complex media for correlation to production of bacteriocin 105b, TSB was determined to be the best complex media for yielding a high quantity of the antimicrobial peptide. Similarly, upon evaluation of nitrogen and carbon sources in growth media, both tryptone and sucrose were found to be advantageous for the production of bacteriocin 105b.

Table 6 shows the results of the cost analysis. Prices were obtained from the VWR Scientific website accessed on September 27, 2015. The total cost of the growth media is determined for 1 L of media. The cost of utilizing TSB is $7.56 while the cost for the tryptone/sucrose/minimal media formulation is $5.05 and the cost for the molasses/trace mineral/minimal media formulation is $1.84. Though TSB appeared to be the superior growth media for production of bacteriocin 105b, its high cost may render production of the antimicrobial peptide using TSB as cost prohibitive. Additionally, although sucrose and tryptone in combination with minimal media would yield sufficient production of bacteriocin 105b, the cost of production may be too high to bear. As precedence for optimization of bacteriocin production has been documented (Mohammed Abdel-Mawgoud *et al*, 2008) and due to the low cost of materials, the molasses/trace mineral/minimal media composition was chosen as an ideal growth media to investigate for optimized production of bacteriocin 105b.

Table 6. Cost Analysis of Growth Media for Production of Bacteriocin 105b

A. Tryptic Soy Broth (TSB)

Component	Supplier	Price/Weight	Actual Use	Cost/L	
TSB media	Hardy Diagnostics	$503.67 / 2 kg	30 g/L	$7.56	
				$7.56	Total Cost

B. Tryptone/Sucrose/Minimal Media

Component	Supplier	Price / Weight	Actual Use	Cost/L	
Tryptone	IBI Scientific	$122.09 / 1 kg	30 g/L	$3.66	
Sucrose	Alpha Aesar	$19.52 / 500 g	5 g/L	$0.20	
Dipotassium Phosphate	VWR Life Science	$128.15 / 1 kg	7 g/L	$0.90	Minimal media
Monopotassium Phosphate	G-Biosciences	$72.89 / 1 kg	2 g/L	$0.15	
Sodium Citrate	Alfa Aesar	$71.64 / 1 kg	0.5 g/L	$0.04	
Magnesium Sulfate	VWR Life Science	$79.16 / 1 kg	0.1 g/L	$0.01	
Ammonium Sulfate	BDH	$44.24 / 500 g	1 g/L	$0.09	
				$5.05	Total Cost

C. Molasses/Trace Minerals/Minimal Media

Component	Supplier	Price / Weight	Actual Use	Cost/L	
Molasses	Golden Barrel Blackstrap	$26.49 / 5 gal	0.026 gal (100 mL)	$0.14	Molasses
Calcium Chloride	VWR	$91.07 / 1 kg	0.5 g/L	$0.05	
Iron (II) Chloride	Alpha Aesar	$451.46 / 250 g	0.2 g/L	$0.36	
Sodium Nitrate	VWR	$41.03 / 1 kg	25 g/L	$1.03	
Manganese Sulfate	VWR	$48.23 / 1 kg	0.25 g/L	$0.01	Trace Minerals
Copper Sulfate	Anachemia	$305.31 / 500 g	0.005 g/L	$0.005	
Zinc Sulfate	VWR	$38.73 / 500 g	0.05 g/L	$0.005	
Dipotassium Phosphate	VWR Life Science	$128.15 / 1 kg	1.4 g/L	$0.18	Minimal media
Monopotassium Phosphate	G-Biosciences	$72.89 / 1 kg	0.4 g/L	$0.03	
Sodium Citrate	Alfa Aesar	$71.64 / 1 kg	0.1 g/L	$0.008	
Magnesium Sulfate	VWR Life Science	$79.16 / 1 kg	0.02 g/L	$0.002	
Ammonium Sulfate	BDH	$44.24 / 500 g	0.2 g/L	$0.009	
				$1.83	Total Cost

To determine the optimal components of molasses media required to produce high yields of bacteriocin 105b, a design matrix was produced using the "Design of Experiments" (DOE) software. This matrix identified several concentrations of the components of the molasses/trace mineral/minimal media formulation for evaluation on the effects on bacteriocin 105b production. The matrix provided guidance on varying the concentration of molasses, iron chloride ($FeCl_2$), calcium chloride ($CaCl_2$) and trace elements to yield statistically relevant results toward the identification of an optimal formulation. Resulting activity of bacteriocins produced from the formulations described in the matrix was evaluated against the target pathogenic microorganism *Bacillus anthracis* Sterne.

The molasses solution was prepared by combining 200 mL of molasses and 200 mL of deionized water. Each solution was exposed to the steam of a hot water bath for approximately 1 h. The apparatus was wrapped in aluminum foil in order to trap the steam. Solutions were then removed, transferred to Nalgene tubes and cooled to room temperature. Once equilibrated to ambient temperature, the vials were centrifuged for 30 min at 10,000 rpm at 4 ºC. The supernatant was removed and stored at 4 ºC until future use.

The solution of minimal media was prepared following the Difco Media Composition Handbook. **Table 7** shows the quantity of each component of minimal media. For these studies, 2 L at 5x concentrated was prepared by combining 70 g dipotassium phosphate, 20 g monopotassium phosphate, 5 g sodium citrate, 1 g magnesium sulfate, 10 g ammonium sulfate and 25 g of sodium nitrate. Deionized water was added until a volume of 2 L was achieved. The solution was stirred until solutes went into solution and then filter sterilized, as minimal media precipitates when exposed to high temperatures.

Table 7. Composition of Minimal Media

Dipotassium Phosphate	7 g/L
Monopotassium Phosphate	2 g/L
Sodium Citrate	0.5 g/L
Magnesium Sulfate	0.1 g/L
Ammonium Sulfate	1 g/L
Sodium Nitrate	25 g/L

The compositions of the trace mineral solutions were based on literature (Mohammed Abdel-Mawgoud *et al.* 2008). The trace mineral solution was composed of manganese sulfate, copper sulfate, zinc sulfate and iron chloride. To investigate the effect of iron chloride on the production of bacteriocin 105b, a base trace mineral solution was prepared to which varying concentrations of iron chloride could be added. Base trace mineral solutions at three concentrations (2x, 1x and 0.5x) were prepared. **Table 8** displays the composition of each base trace mineral solution.

Table 8. Composition of Base Trace Mineral Solutions

2x Concentrated Solution:	1x Concentrated Solution	0.5x Concentrated Solution
DI Water: 500 mL Manganese Sulfate: 0.5 g Copper Sulfate: 0.01 g Zinc Sulfate: 0.1 g	DI Water: 250 mL 2x Soln.: 250 mL	DI Water: 250 mL 1x Soln.: 250 mL

Solutions of the base trace mineral solution were combined with either 5 g or 10 g of iron chloride to yield a concentration of 0.1 g/L or 0.2 g/L of iron chloride respectively. **Table 9** shows the quantity of deionized water (DI water), concentration of base trace mineral (BTM) solution referenced in **Table 10** and quantity of iron chloride combined.

Table 9. Preparation of Trace Mineral Solutions with Varying Concentrations of Iron Chloride

Solution 1	Solution 2	Solution 3	Solution 4	Solution 5	Solution 6
DI Water: 450 mL 2x BTM: 50 mL Iron Chloride: 5 g (0.1 g/L)	DI Water: 450 mL 2x BTM: 50 mL Iron Chloride: 10 g (0.2 g/L)	DI Water: 450 mL 1x BTM: 50 mL Iron Chloride: 5 g (0.1 g/L)	DI Water: 450 mL 1x BTM: 50 mL Iron Chloride: 10 g (0.2 g/L)	DI Water: 450 mL 0.5x BTM: 50 mL Iron Chloride: 5 g (0.1 g/L)	DI Water: 450 mL 0.5x BTM: 50 mL Iron Chloride: 10 g (0.2 g/L)

The DOE matrix experiment to determine the optimal composition of molasses media for the production of bacteriocin 105b was carried out by combining varying amounts of each component to be tested. **Table 10** displays the composition of each sample evaluated. For each sample, the corresponding volume of molasses, mass of calcium chloride, trace mineral solution with iron chloride and volume of water is shown. The volume of minimal media added was constant at 20 mL. Each sample of molasses media was prepared by combining each component following the necessary formulation represented in **Table 10**.

Table 10. Composition of Samples for DOE Experiment for Determining Optimal Formulation for Microorganism Growth

Sample #	**Molasses (mL)**	**$CaCl_2$ (g/L)**	**Trace Mineral Solution $FeCl_2$**			**Minimal Media (mL)**	**DI Water**
			Soln. no.	$FeCl_2$ (g/L)	[BTM] (x)		
1	16	0.05	3	0.1	1	20	54
2	10	0.1	4	0.2	1	20	60
3	10	0.1	3	0.1	1	20	60
4	10	0.05	1	0.1	0.5	20	60
5	16	0.1	2	0.2	0.5	20	54
6	10	0.1	5	0.1	2	20	60
7	10	0.1	1	0.1	0.5	20	60
8	16	0.05	5	0.1	2	20	54
9	16	0.1	6	0.2	2	20	54
10	16	0.1	2	0.2	0.5	20	54
11	10	0.1	4	0.2	1	20	60
12	16	0.05	4	0.2	1	20	54
13	16	0.1	2	0.2	0.5	20	54
14	16	0.05	6	0.2	2	20	54
15	16	0.1	1	0.1	0.5	20	54
16	10	0.05	4	0.2	1	20	60
17	16	0.05	3	0.1	1	20	54
18	10	0.05	4	0.2	1	20	60
19	10	0.1	5	0.1	2	20	60
20	16	0.05	6	0.2	2	20	54
21	16	0.05	5	0.1	2	20	54
22	10	0.05	1	0.1	0.5	20	60
23	10	0.1	1	0.1	0.5	20	60
24	10	0.05	6	0.2	2	20	60

Following preparation of the DOE matrix formulations of molasses media, each was inoculated with a single colony of the bacteriocin 105b producer strain. The flasks were incubated for 20 h in a shaker incubator at 37 °C. After incubation, a 1.5 mL aliquot of the culture was removed and centrifuged for 25 min at 4 °C. One milliliter of the supernatant was removed, filtered through a 0.22 µm filter and concentrated using a SpeedVac until the final volume decreased to 200-350 µL. To ensure all samples in a given run were at the same volume, water was added as necessary to increase the volume.

Initially, evaluation of the yield of bacteriocin 105b produced in the DOE matrix formulations of molasses media was performed using a time kill-kinetic assay against the target strain, *Bacillus anthracis* Sterne. In this assay, it is expected that an effect will be observed on the target's kinetic growth curve in the presence of an antimicrobial agent. The greater the concentration of antimicrobial agent (the bacteriocin) present, the greater the effect on growth as observed as a greater lag time necessary for the population of the target microorganism to initiate exponential growth. In some instances, the quantity of antimicrobial agent present is so great that growth of the target is never observed. Thus, if a DOE matrix formulation for molasses media yielded a high production or activity of bacteriocin 105b, it is expected that the lag time for growth of the target microorganism would be greater than for a DOE matrix formulation that yielded lower production or activity of bacteriocin 105b. The resulting lag time observed in the kinetic assay can be input into the DOE software to provide statistical analysis on the molasses media formulation that yields the optimal production of bacteriocin 105b.

For the kinetic assay, the target strain was prepared by inoculating a single colony of fresh culture of *Bacillus anthracis* Sterne from a streak plate into 10 mL of nutrient broth. The culture was incubated on a shaker incubator for 4 h at 37 °C or until the OD measured between 0.5-1 AU at 600 nm to ensure a target concentration of $10^7 – 10^8$ cells. Serial dilutions of concentrated bacteriocin 105b representing concentrated (100%), 2x dilute (50%) and 4x dilute (25%) solutions were evaluated for their effect on activity of the target strain. The dilutions of bacteriocin 105b were incubated with 150 µL of the target culture in a microtiter plate. The microtiter plate was incubated overnight at 37 °C and the growth of the target microorganism was assessed using the Omnilog microtiter plate reader. For a positive control, dilutions of nisin, an alternative bacteriocin that has demonstrated activity against the target, were evaluated. The concentration of the 100% concentrated nisin sample was 5 mg/mL. For a negative control, the kinetic growth of target in the absence of antimicrobial peptide was assessed.

Figure 16 shows representative results of the kinetic assay. **Figure 16A** shows the kinetic growth curve for the target *Bacillus anthracis* Sterne in the absence of antimicrobial peptide. As expected, the OD of the target increases over time, signifying the growth of the population. **Figure 16B** shows representative data for the target grown in the presence of nisin, another bacteriocin previously demonstrated to exhibit antimicrobial activity on the target. At the three dilutions tested, no growth of the target is observed, indicative of nisin completely inhibiting the growth of the target as expected. **Figure 16C** shows the results of the time kill-kinetic assay for the target in the presence of 100%, 50% and 25% concentrated cell extract from the bacteriocin 105b producer organism grown in the DOE Matrix Molasses Formulation from Sample 1 (see **Table 10**). The results of the kinetic assay with bacteriocin 105b in Sample 1 of the DOE Matrix Molasses Formulation, as well as the kinetic assays of the additional DOE Matrix Molasses

Formulation Samples was inconclusive. As observed in **Figure 16C**, the high absorbance observed from the components of the molasses media prevented analyzation of the kinetic growth curve of the target culture in the presence of bacteriocin 105b produced in the DOE matrix molasses media formulations. Steps taken to alleviate the effects of the components of the molasses formulations on absorbance readings by the spectrophotometer include heating the molasses solution in a steam bath followed by centrifugation as detailed previously. Additionally, gravimetric chromatography was performed on cell extracts collected from each molasses media formulation, but the activity of the bacteriocin was not preserved. Because of this, evaluation of the production of bacteriocin 105b as characterized by activity using the kinetic growth curve was not possible.

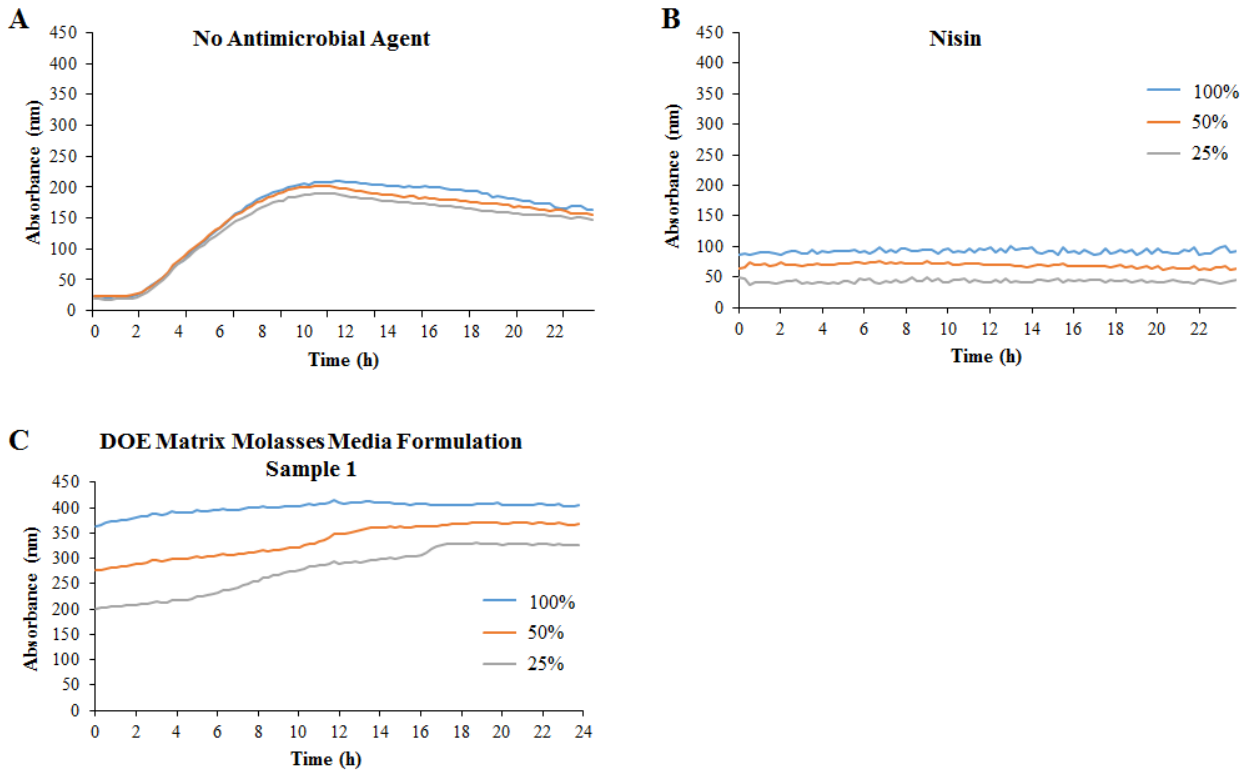


Figure 16. Representative kinetic assay data. A.) W. B.) In the presence of nisin at three different concentrations. C.) In the presence of three different concentrations of bacteriocin 105b in DOE Matrix Molasses Media Formulation sample 1.

An alternative metric was sought to evaluate the effects of the different molasses growth media formulations on the production and activity of bacteriocin 105b for input into the DOE software to facilitate statistical analysis. In parallel to the kinetic assay, activity drop tests were performed using the 100% and 25% concentrated cell extract of each DOE Matrix Molasses Media Formulation. The target overlay was prepared by combining a 20 µL aliquot of a stock of *Bacillus anthracis* Sterne grown to an OD of 1 with overlaid plate 7 mL of nutrient soft agar and poured over a media plate. A 6 µL aliquot of the 100% and 25% concentrated cell extract from each DOE Matrix Molasses Media Formulation was dropped on the target overlay and incubated overnight at 37 °C. Antimicrobial activity was determined by the presence of a zone of clearing, suggesting the presence of bacteriocin 105b. The diameter of the zone of clearing is expected to

correlate to the presence or activity of bacteriocin 105b such that a greater diameter correlates to, a greater activity or quantity of the antimicrobial peptide. Thus, analysis of the diameter of the zones of clearing observed for the activity drop test were applied as metrics to assess the molasses media formulations designed by the DOE software and provide data to be input into the software for statistical analysis.

Zones of clearing resulting from the undiluted drops were found to yield the greatest distinction for the inhibition of the cell extracts against the target. If a zone of clearing was present, its diameter was measured once using a standard ruler and recorded. For each molasses media formulation, the activity drop test was repeated four times to accumulate an average of diameters and the standard deviation was calculated to account for error. Data was normalized for each run.

Figure 17 shows the results of assessing the activity of bacteriocin 105b produced in the DOE matrix molasses media formulations tested by employing measurements of the zones of clearing obtained from the activity drop test assay. The x-axis represents the sample number corresponding to the molasses media formulation in **Table 10** for the DOE matrix, while the y-axis represents normalized activity as determined from measurements of the zones of clearing observed. The height of each bar is indicative of the antimicrobial activity observed for the cell extract from each molasses media formulation. The higher the bar, the greater the zone of clearing observed which may be attributed to a greater quantity or activity of bacteriocin 105b present. Based on the size of the error bars, several of the runs were inconsistent, which suggests that the production of bacteriocin 105b in these molasses media formulations is variable. Additionally, several molasses media formulations exhibited poor activity, suggesting that the production of bacteriocin 105b in these DOE matrix compositions was low. For example, the cell extracts from samples 6 and 13 yielded small zones of clearing and inconsistent results whereas the cell extracts from samples 16 and 17 generated larger zones of clearing and more consistent results as observed from the smaller error bars. This observation alludes to a greater quantity of bacteriocin 105b being produced or exhibiting greater activity when the producer organism is grown in molasses media formulations composed of samples 16 and 17 as opposed to samples 6 and 13.

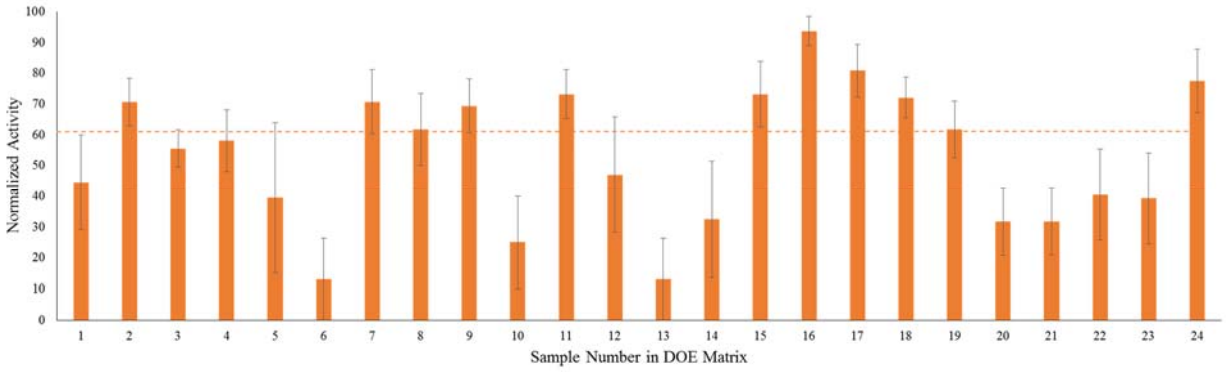


Figure 17. Average activity of bacteriocin 105 in DOE Matrix Molasses Media Formulations

At the time of this report, the data has not been input into the DOE software to provide statistical data on trends for the optimal composition of molasses media to produce the greatest quantity of

bacteriocin 105b. Though preliminary analysis of the data suggests that several trends are apparent when considering molasses media formulations that provided more than 60% of activity. The dashed horizontal line in **Figure 17** represents 60% activity. Samples whose average activity exceeded the 60% threshold were considered successful performers, while samples whose average activity did not meet the 60% threshold were considered poor performers. Using this criteria, molasses media formulations that were judged good performers were samples 2, 7, 9, 11, 15, 16, 17, 18, and 24. Molasses media formulations that were deemed poor performers were 1, 3, 4, 5, 6, 10, 12, 13, 14, 20, 21, 22 and 23. Applying this simple activity threshold to distinguish between activity of the samples tested indicates that less than half of the molasses media formulations were successful.

Tables 11 displays the compositions of the molasses media formulations for samples whose activity averaged above the 60% activity threshold and highlights several trends. As illustrated in **Table 11**, 67% of the samples included 10 mL of molasses (highlighted in yellow) and 67% included a concentration of iron chloride equal to 0.2 g/L (highlighted in blue). This suggests that the lower concentration of molasses and higher concentration of iron chloride evaluated are advantageous to the activity, which is expected to relate to the quantity, of bacteriocin 105b present in the cell extract for these formulations. Additionally, 56% of the samples were comprised of 1x concentration of base trace mineral solutions (highlighted in orange), indicating that this concentration may also contribute to the observation of greater activity. The concentration of calcium chloride present in the molasses media formulations does not appear to significantly influence the activity of bacteriocin 105b in the resulting cell extracts. Of the nine samples considered to be successful, five were comprised of 0.1 g/L of $CaCl_2$ while four included 0.05 g/L of $CaCl_2$. The results of this preliminary analysis indicate that concentrations of molasses, iron chloride and trace minerals in the molasses media formulations effect the generation of bacteriocin 105b as observed through greater antimicrobial activity against the target.

Table 11. Molasses Media Formulations that Exhibit Activity Greater than 60%

	Components			
Sample #	Molasses (mL)	$CaCl_2$ (g/L)	$FeCl_2$ (g/L)	[BTM] (x)
2	10	0.1	0.2	1
7	10	0.1	0.1	0.5
9	16	0.1	0.2	2
11	10	0.1	0.2	1
15	16	0.1	0.1	0.5
16	10	0.05	0.2	1
17	16	0.05	0.1	1
18	10	0.05	0.2	1
24	10	0.05	0.2	2

Conversely, **Table 12** displays the molasses media formulations for the poor performer samples that did not meet the 60% activity threshold and highlights several trends. For example, 62% of the samples were comprised of 16 mL of molasses, the highest molasses concentration tested (highlighted in yellow). The impact of the concentration of calcium chloride and iron chloride was less distinguishable with 0.05 g/L of $CaCl_2$ and 0.1 g/L of $FeCl_2$ being the concentrations of these compounds for 7 out of the 13 total poor performers. Regarding the concentration of base trace mineral solution, 46% of the samples included 0.5x concentration (highlighted in orange) suggesting that this lowest concentration of trace mineral solution tested may correlate to greater production as visualized by activity of bacteriocin 105b.

Table 12. Molasses Media Formulations that Exhibit Activity Less than 60%

	Components			
Sample #	Molasses (mL)	$CaCl_2$ (g/L)	$FeCl_2$ (g/L)	[BTM] (x)
1	16	0.05	0.1	1
3	10	0.1	0.1	1
4	10	0.05	0.1	0.5
5	16	0.1	0.2	0.5
6	10	0.1	0.1	2
10	16	0.1	0.2	0.5
12	16	0.05	0.2	1
13	16	0.1	0.2	0.5
14	16	0.05	0.2	2
20	16	0.05	0.2	2
21	16	0.05	0.1	2
22	10	0.05	0.1	0.5
23	10	0.1	0.1	0.5

While the statistical analysis afforded by the DOE software will provide more detailed evaluation of the data, this preliminary analysis provides insight into the necessary components for high production of bacteriocin 105b in molasses media. The crude analysis discussed above indicates that lower concentration of molasses (10 mL) is better than higher concentration of molasses (16 mL) for the production of bacteriocin 105b in the molasses growth media formulations. The concentration of calcium chloride appears to be inconsequential. Further, the results indicate the production of bacteriocin 105b from the producer organism in molasses media favors the presence of 1x concentration of base trace mineral solution and may be inhibited by 0.5x concentration of base trace mineral solution. These data should be used as a stepping stone for carrying out more detailed analysis of the DOE Matrix Molasses Media Formulations through additional assays to confirm these preliminary findings. Additionally, establishing the quantity of bacteriocin 105b produced for each molasses media formulation would elucidate the relationship between the quantity of antimicrobial peptide generated and the magnitude of antimicrobial activity observed.

Summary of Task V

In this task, the production of bacteriocin 105b was evaluated in several growth media to investigate the conditions for optimal yield of the antimicrobial peptide. Because bacteriocin 105b exhibits targeted activity against the surrogate strain of anthrax, the antimicrobial peptide has great promise for use in future applications to protect the Warfighter. To realize these applications, the yield of the peptide acquired from the bacteriocin 105b producer strain must be optimized to reduce cost and labor for large-scale applications. Evaluation of the literature suggests that the composition of the media, including the carbon and nitrogen source present, can alter the generation of bactericions (Mahrous *et al*. 2013, Mataragas *et al*. 2004). Several complex media were evaluated and TSB was found to generate the highest yield of bacteriocin 105b as indicated by activity studies. Additionally, nitrogen and carbon sources were investigated. The results of these studies indicate that tryptone and peptone yield superior production of bacteriocin 105b, while the carbon sources that generate optimal production of bacteriocin 105b are dextrose, mannose, molasses and sucrose.

To account for the cost of bacteriocin 105b production, a cost analysis of employing TSB, tryptone/sucrose/minimal media and molasses/trace mineral/mineral media was conducted. The molasses media formulation was determined to be significantly cheaper. Based on this cost analysis and a recent trend to employ byproducts from the sugar industry including low-cost sugars and molasses (Metsoviti *et al*. 2011), the molasses media formulation was evaluated to optimize the production of bacteriocin 105b as indicated via activity assays. Using the DOE software, a matrix of variables at a range of concentrations was produced. The molasses formulations were prepared and *Bacillus subtilis* 105b, the producer strain of bacteriocin 105b, was grown in each. The resulting cell extracts were evaluated for activity using zones of clearing from activity drop tests to assess for the magnitude of production of the antimicrobial peptide. Although the data has not been analyzed by the DOE software, preliminary observations suggest that the concentration of molasses and base trace mineral solution that comprise the formulation may affect the production of bacteriocin 105b.

Further studies are required in order to comprehensively evaluate the production of bacteriocin 105b in molasses media. For example, the quantity of bacteriocin 105b generated in these formulations must be determined in order to ascertain that the magnitude of activity observed is due to the yield of antimicrobial peptide present. Additionally, replication of analysis of the DOE Matrix Molasses Media Formulations is required to generate more consistent results. This data should be analyzed by the DOE software to provide statistical evidence for an optimized molasses formulation. Further, once a high yield of the bacteriocin is established, the purification process required to remove impurities from the growth media as well as cellular debris must be considered. Although molasses media may provide the greatest cost benefit for the production of bacteriocin 105b, the potential extensive purification process required to generate a pure or semi-pure product may render the growth media unfeasible.

Conclusions and Recommendations

In future studies regarding bacteriocin 105b, the antimicrobial peptide should be sequenced to ascertain if the antimicrobial peptide is novel or has been previously characterized in literature. The current allure of developing bacteriocin 105b to be a targeted antimicrobial agent is its uniqueness. Elucidating the sequence of bacteriocin 105b is necessary to determine if this antimicrobial peptide has been newly discovered or is a bacteriocin that has already been well characterized. If it is found that bacteriocin 105b is not novel, the work detailed in this effort may still provide insight into the antimicrobial properties and the production/purification schemes that were evaluated, which will be of interest to the scientific community.

Future work must also account for the yield of bacteriocin 105b throughout the studies detailed in this report. Manipulation of the growth media as well as purification protocols are expected to have significant effects on the quantity of bacteriocin 105b generated. As a result, in order to comprehensively evaluate the studies detailed in this report, the yield of the antimicrobial peptide must be considered. While the BCA would afford knowledge of the total protein concentration (also relevant), advanced techniques such as HPLC and/or FPLC should be conducted to determine accurate yield of bacteriocin 105b. Additionally, HPLC and/or FPLC would further characterize the purity of the antimicrobial peptide.

It is envisioned that the use of bacteriocin 105b will be in a textile that demonstrates antimicrobial activity to protect the Warfighter from pathogenic bacterial threats. To accomplish this goal, the stability of bacteriocin 105b in a textile must be evaluated and supplemented as necessary to ensure the antimicrobial peptide remains active. Long-term stability and activity of bacteriocin 105b should be established to determine the effects of potential storage conditions of the antimicrobial textile in an employable environment. Additionally, the activity of bateriocin 105b against commensal flora, as well as the identity of any remaining impurities in the extract of bacteriocin 105b must be evaluated to ensure the safety of the Warfighter.

The efforts detailed in this report arising from the BaCTeRIA project provide a substantial foundation in the identification, development and processing of an antimicrobial peptide that has demonstrated great promise for application in a novel construct to provide antimicrobial protection to the Warfighter.

This document reports research undertaken at the U.S. Army Natick Soldier Research, Development and Engineering Center, Natick, MA, and has been assigned No. NATICK/TR- 17/017 in a series of reports approved for publication.

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Appendix. Results of Physical and Biochemical Analysis to Determine Identity of Environmental Isolates

#	Name	Organism Identity	Gram	Shape	Spore	Swollen	Catalase	Starch Hyd	Oxidase	Glucose Ferm	VP	Novo-biocin	Citrate	Growth in 6.5% Na	Nitrate Reduction
59	FD2 a	*Bacillus*	+	Rod	Yes	+	+	+		X	-				
60	FD3 a	*Bacillus*	+	Rod	Yes	+	+	+			-			-	
61	FD6 a	*Bacillus*	Variable	Rod	Yes	+	+	+	+	-	X				
62	FD6 b	*Bacillus*	+	Rod	Yes		+	+							
63	FD6 c	*Bacillus*	+	Rod	Yes	-	+	+			-		X		
64	FD6 d	*Cupriavidus*	-	Rod					+	-					
65	FD6 e	*Bacillus*	+	Rod	Yes		+	+							
66	FD6 f	*Bacillus*	+	Rod	yes	-	+	-							
67	FD6 g	*Bacillus*	+	Rod	yes	-	+	-							
68	FD6 h	*Cupriavidus*	-	Rod					+	-					
69	FD6 i	*Bacillus*	+	Rod	Yes	+	+	-							
70	FD6 j	*Cupriavidus*	-	Rod					+	-					+
71	FD6 k	*Bacillus*	+	Rod	Yes			+							+ beforeZn
72	FD6 l	*Bacillus*	+	Rod	Yes	+	+	-							
73	FD6 m	*Bacillus*	+	Rod	Yes	-	+	+			-		X		
74	FD6 n	*Cupriavidus*	-	Rod					+	-					
75	FD6 o	*Pseudomonas*	-	Rod	maybe				+	-					-
76	FD6 p	*Cupriavidus*	-	Rod					+	-					X
77	FD6 q	*Cupriavidus*	Variable	Rod	Yes		+	-							
78	FD6 r	*Cupriavidus*	-	Rod			+		+	-					
79	FD6 s	*Bacillus*	+	Rod	yes	+			+	-					
80	FD6 t1	*Bacilus*	+	Rod	Yes	-	+	-							
81	FD6 t2	*Bacillus*	Variable	Rod	Yes	yes	+	+			-			-	
82	FD6 u	*Bacillus*	+	Rod	Yes	yes	+	+			-				
83	FD6 v	*Bacillus*	+	Rod	Yes	-	+	-						-	
84	FD6 w	*Bacillus*	+	Rod	Yes	-	+	-							-
85	FD6 x1	*Bacillus*	+	Rod	Yes		+	+			+		X		

86	FD6 x1	*Bacillus*	Variable	Rod	Yes	-	-	+			-				+ before Zn
87	FD6 y	*Bacillus*	Variable	Rod	Yes		+	+			-				
88	FD6 z	*Cupriavidus*	-	Rod											
89	FD6 aa	*Cupriavidus*	-	Rod	maybe		+		+	-					
90	FD6 bb	*Bacillus*	+	Rod	Yes		+	-							+
91	FD6 cc	*Bacillus*	variable	Rod	Yes	-	+	-	+	-				-	
92	FD6 dd		Variable	rod	yes	yes	+					X			
93	FD6 ee	*Bacillus*	+	Rod	Yes		+	+						+	
94	FD6 ff	*Bacillus*	Variable	Rod	no	X	+	-							+ before Zn; frothy
95	FD6 gg		-	Rod					+	-					
96	FD6 hh	*Bacillus*	+	Rod	Yes	X	+	-						-	
97	FD6 ii	*Cupriavidus*	-	Rod			+		+	-					
98	FD6 jj	*Cupriavidus*	-	Rod					+	-					
99	FD7 a	*Staph*	+	Cocci			+								
100	FD7 b	*Staph*	+	Cocci			+					X			+ after Zn
101	FD7 c	*Staph*	+	Cocci			+					+			
102	FD7 d	*Staph*	+	Cocci			+			-		+			
103	FD7 e	*Staph*	+	Cocci			+					+			
104	FD7 f	*Staph*	+	Cocci			+					+			
105	FD7 g	*Staph*	+	Cocci			+					X			
106	FD7 h	*Staph*	+	Cocci			+					+			
107	FD7 i	*Staph*	+	Cocci			+					+			
108	FD7 j	*Staph*	+	Cocci			+					+			
109	FD7 k	*Staph*	+	Cocci			+								
110	FD7 l	*Staph*	+	Cocci			+					+			
111	FD7 m	*Staph*	+	Cocci			+					X			
112	FD7 n	*Staph*	+	Cocci			+								
113	FD7 o	*Staph*	+	Cocci			+					+			+ w/ gas
114	FD7 p	*Micrococcus*	+	Cocci			+			-				-	

115	FD7 q	*Staph*	+	Cocci			+					+			+ after Zn; frothy
116	FD7 r	*Staph*	+	Cocci			+					+			
117	FD7 s	*Staph*	+	Cocci			+					+			
118	FD7 t1	*Staph*	+	Cocci			+					+			
119	FD7 u	*Staph*	+	Cocci			+			-					
120	FD7 v	*Staph*	+	Cocci			+					+			
121	FD7 w	*Staph*	+	Cocci			+					+			
122	FD7 x	*Staph*	+	Cocci			+					+			
123	FD7 y	*Staph*	+	Cocci			+					+			
124	FD7 z	*Staph*	+	Cocci			+					+			
125	FD7 aa	*Staph*	+	Cocci			+					+			
126	FD7 bb						+								
127	FD7 cc	*Micrococcus*	+	Cocci			+			-					
128	FD8 a	*Bacillus*	+	Rod	Yes		+	+							
129	FD8 b	*Bacillus*	Variable	Rod	Yes	+	+	-							
130	FD8 c	*Micrococcus*	+	Cocci			+			-				+	
131	FD8 d	*Bacillus*	+	Cocci			+					-			
132	FD8 e	*Micrococcus*	+	Cocci			+			+					
133	FD8 f	*Aquaspirillum*	-	Rod					+	-					
134	FD8 g	*Bacillus*	Variable	Rod	Yes	+	+	+	+	+	-	+			
135	FD8 h	*Bacillus*	+	Rod	Yes		+	+			X				
136	FD8 i	*Bacillus*	+	Rod	Yes	+	+	-						-	
137	FD8 j	*Bacillus*	Variable	Rod	Yes	maybe	+	+			-				-
138	FD8 k														
139	FD8 l	*Micrococcus*	+	Cocci			+			-				+	
140	FD8 m	*Bacillus*	+	Rod	Yes	X	+	-							
141	FD8 n	*Micrococcus*	Variable	Cocci			+			-					
142	FD8 o	*Bacillus*	+	Rod	Yes				+	-				-	
143	FD8 p	*Bacillus*	+	Rod	yes	yes	+	maybe	+	-					+ before Zn

144	FD8 q	*Bacillus*	+	Rod	Yes		+	-							
145	FD8 r	*Brevibacillus*	Variable	Rod					+	-					
146	FD8 s														
147	FD8 t	*Micrococcus*	+	Cocci			+			-					
148	FD8 u	*Bacillus*	+	Rod	Yes		+	+							
149	FD8 v	*Bacillus*	+	Rod	Yes	+	+	+	+	+	-			-	
150	FD8 w	*Staph*	+	Cocci			+					+			
151	FD8 x	*Bacillus*	+	Rod	Yes		+	lost plate							
152	FD10 a	*Bacillus*	+	Rod	Yes	-	+	+			-				X
153	FD10 b	*Pseudomonas*	-	Rod			+		+	-					-
154	FD10 c	*Bacillus*	+	Rod	Yes		+	+							
155	FD10 d	*Bacillus*	+	Rod					+	-					
156	FD10 e	*Staph*	+	Cocci			+					+			
157	FD10 f	*Bacillus*	+	Rod	Yes		+	+							
158	FD10 g		-	Rod					+	+					
159	FD10 h	*Staph*	+	Cocci			+					+ ?			
160	FD10 i	*Nocardia?*	-	Mixed	yes	not swollen	+		Positive						
161	FD11 a	*Bacillus*	+	Rod	Yes	X	+	+			-				+ after Zn
162	FD11 b	*Bacillus*	+	Rod	Yes	-	+	-							
163	FD11 c	*Bacillus*	+	Rod	Yes	+	-	+			-				
164	FD11 d	*Bacillus*	+	Rod	Yes	-	+	+			-				
165	FD11 e	*Bacillus*	+	Rod	Yes	maybe	+	+			-				
166	FD11 f	*Bacillus*	Variable	Rod	Yes	-		+	+	+	-				
167	FD11 g	*Bacillus*	+	Rod	Yes		+	-							
168	FD12 a	*Bacillus*	+	Rod	Yes	+	+	+			-				X
116a	FD7ra	*Bacillus*	X	X	yes	+	+								
118b	FD7 t2		+	Cocci			+								
60b	FD 3b	*Bacillus*	+	rod	Yes	yes	+	-			+				
71b	FD6Kb		+	cocci or rod	no	?	+								